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Oxidative Stress and Nuclear Factor-κB Activation

A REASSESSMENT OF THE EVIDENCE IN THE LIGHT OF RECENT DISCOVERIES

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ABSTRACT. Nuclear factor-κB (NFκB) is a transcription factor with a pivotal role in inducing genes involved in physiological processes as well as in the response to injury and infection. A model has been proposed whereby the diverse agents that activate NFκB do so by increasing oxidative stress within the cell. Activation of NFκB involves the phosphorylation and subsequent degradation of an inhibitory protein, IκB, and recently many of the proximal kinases and adaptor molecules involved in this process have been elucidated. Additionally, we now understand in detail the NFκB activation pathway from cell membrane to nucleus for interleukin-1 (IL-1) and tumour necrosis factor (TNF). This review revisits the evidence for the oxidative stress model in light of these recent findings, and finds little in the new information to rationalise or justify a central role for oxidative stress in NFκB activation. We demonstrate that much of the evidence for the involvement of oxidative stress is either specific to a stimulus in a particular cell line or open to reinterpretation. In particular, the activation of NFκB by hydrogen peroxide is cell-specific and distinct from physiological activators such as IL-1 and TNF, while inhibition by antioxidants, also found to be cell- and stimulus-specific, can involve diverse and unexpected targets which may be distinct from redox modulation. We conclude that in most cases the role of oxidative stress in NFκB activation is at best facilitatory rather than causal, if a role exists at all. In addition, other evidence suggests a role for lipid peroxides in pathways where such a role exists. In future, when a role for oxidative stress in a pathway is postulated, the challenge will be to show which particular kinases or adaptor molecules, if any, are redox-modulated. BIOCHEM PHARMACOL **59**;1:13–23, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. NFκB; oxidative stress; antioxidants; interleukin-1; tumour necrosis factor; signal transduction

Since its discovery more than a decade ago, the inducible, higher eukaryotic transcription factor NFκB† has been shown to have a pivotal role in the regulation of many genes involved in immune and inflammatory responses (reviewed in [1]). In addition, NFκB has been implicated as important in an ever-expanding list of processes which to date include reactivation and replication of many viruses, embryonic development, control of cell proliferation and apoptosis, neuronal development, and neurodegeneration [2–4]. It is thus not surprising that NFκB has been found to be activated in many cell types in response to a broad range of stimuli and conditions, which typically include viral and bacterial products, T- and B-cell mitogens, inflammatory cytokines such as IL-1 and TNF, intracellular stresses such as endoplasmic reticulum protein overload, and extracellular stresses as diverse as UV light, H_2O_2, cigarette smoke, and asbestos fibres [2, 5–7]. At present, the list of NFκB inducers is expanding monthly. It can therefore be concluded that NFκB represents a 'switch' which when triggered is a very efficient system for regulating gene expression. The genes which are induced play roles in physiological responses during development and also in the response to injury and infection.

NFκB exists in a latent form in the cytoplasm of unstimulated cells comprising a transcriptionally active dimer bound to an inhibitor protein, IκB. The currently known subunit members of the NFκB family in mammals are p50, p65 (RelA), c-Rel, p52, and RelB, while multiple mammalian forms of IκB also exist, namely IκBα, β, γ (p105), δ (p100) and ε, and Bcl-3 [3]. The mechanism whereby diverse stimulants lead to the activation of NFκB has been a subject of intense and exciting research. Most work has focused on the p50/p65 dimer, the predominant form of NFκB activated in many cells, and its association with IκBα. It is now known that upon stimulation with many NFκB inducers, IκBα is rapidly phosphorylated on two serine residues (S32 and S36), which targets the inhibitor protein for ubiquitination and subsequent degradation by the 26 S proteasome [8]. Recently, a specific E3 ligase that recognises doubly phosphorylated IκBα has been identified [9]. The released NFκB dimer can then translo-

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† *Abbreviations:* NFκB, nuclear factor-κB; BHA, butylated hydroxyanisole; DDTC, diethyldithiocarbamate; DFO, desferrioxamine; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; IκB, inhibitor binding protein κB; IKK, IκB kinase; IL-1, interleukin-1; 5-LOX, 5-lipoxygenase; LPS, lipopolysaccharide; NAC, *N*-acetylcysteine; NEMO, NFκB essential modulator; NIK, NFκB-inducing kinase; MEKK, mitogen-activated protein kinase/ERK kinase kinase; PDTC, pyrrolidine dithiocarbamate; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SOD, superoxide dismutase; TAK1, transforming growth factor β-activated kinase 1; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; and TNF, tumour necrosis factor.

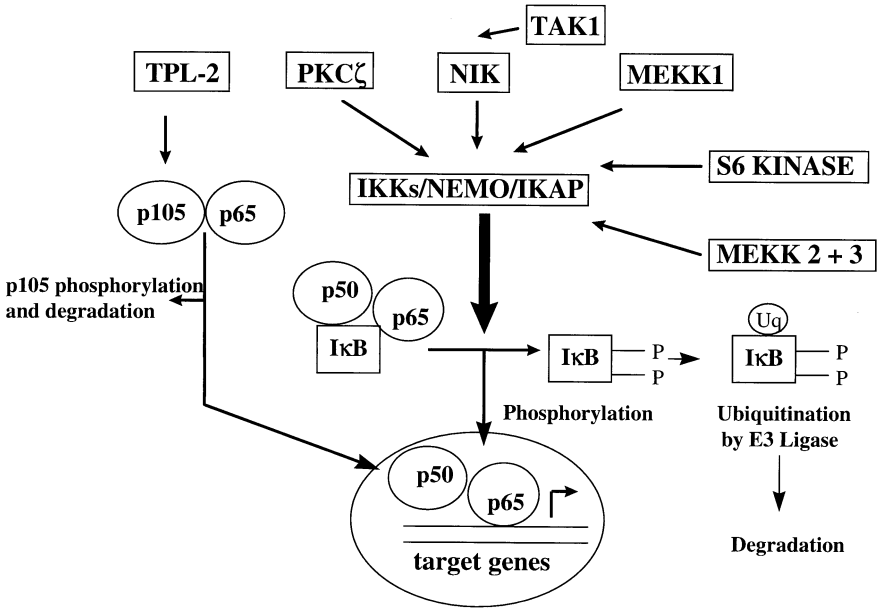

FIG. 1. Mechanisms of regulating NFκB activity. Extracellular stimuli activate signal transduction pathways leading to the activation of kinases that ultimately stimulate IKKs in the IKK signalsome. NEMO and the IKK complex-associated protein (IKAP) are essential for IKK activity. Once phosphorylated by the IKKs, IκB is ubiquitinated by a specific E3 ligase, facilitating its degradation. Released p50/p65 can then translocate to the nucleus and affect target genes. Kinases recently shown to act upstream of IKK include NIK, MEKK1, 2 and 3, TAK1, protein kinase Cζ, and S6 kinase. An alternative route to NFκB activation, independent of IκB, involves the kinase TPL-2 (tumour progression locus 2 kinase) which can phosphorylate p105, leading to its processing into p50.

cate to the nucleus and activate target genes by binding with high affinity to κB elements in their promoters. The phosphorylation and degradation of IκBα are tightly coupled events, so it was always likely that agents that activate NFκB do so by stimulating a specific IκBα kinase, or alternatively by inactivating a particular phosphatase.

PATHWAYS TO NFκB ACTIVATION DELINEATED

Recently, there have been a flurry of papers describing and characterising two IκB kinases, termed IKKα and IKKβ (reviewed in [10]). IKKα and β have been shown to be activated by important inducers of NFκB such as IL-1 and TNF, to specifically phosphorylate S32 and S36 of IκBα, and to be crucial for NFκB activation by these cytokines [11–15]. The IKKs are part of a larger multiprotein complex called the IKK signalsome, which contains IKAP (IKK complex-associated protein) and NEMO (also called IKKγ), also shown to be essential for NFκB activation [16–18]. IκBβ can be phosphorylated on two equivalent serine residues, S19 and S23, by both IKKα and IKKβ [11–15], and there are also homologous serine residues in IκBε (S157 and S161) which are important for NFκB activation [19], suggesting that the pathways to the release of NFκB from these inhibitory subunits will be similar to IκBα. Many upstream activators and regulators of IKK activity have already been identified, including NIK [20, 21], MEKK1 [21–23], TAK1 [24, 25], protein kinase Cζ [26], MEKK2 and 3 [27], and S6 kinase [28]. The range of kinases that activate IKK activity is likely to increase, and this may explain how diverse stimulants can all activate NFκB. An alternative route to NFκB, independent of IκBα, β, and ε has also been recently elucidated. The NFκB subunit p50 is translated as a precursor, p105, which can complex to and inactivate p65. TNF has recently been shown to induce p105 processing via its phosphorylation by the kinase TPL-2 (tumour progression locus 2 kinase) [29]. It therefore appears likely that multiple pathways can regulate NFκB, most of which lead to IκB phosphorylation via the IKK-containing signalsome. It is possible that different signalsomes will exist, each containing IKKs, but with different upstream kinases. The current model of how NFκB activation is regulated is illustrated in Fig. 1.

THE OXIDATIVE STRESS MODEL OF NFκB ACTIVATION

An early question in the field of NFκB research concerned the mechanism by which such a diversity of agents might activate NFκB, given that the requirements for activation were clear early on (i.e. the specific phosphorylation of an IκB). A model was proposed whereby diverse agents all activated NFκB by causing oxidative stress [30]. Oxidative stress is defined as an increase in intracellular ROS such as H_2O_2, superoxide (O_2^-), or hydroxyl radical ($\cdot OH$). This hypothesis was based on four main lines of evidence, illustrated in Fig. 2. First, direct addition of H_2O_2 to culture medium activates NFκB in some cell lines [31, 32]. Second, in some cell types ROS have been shown to be increased in

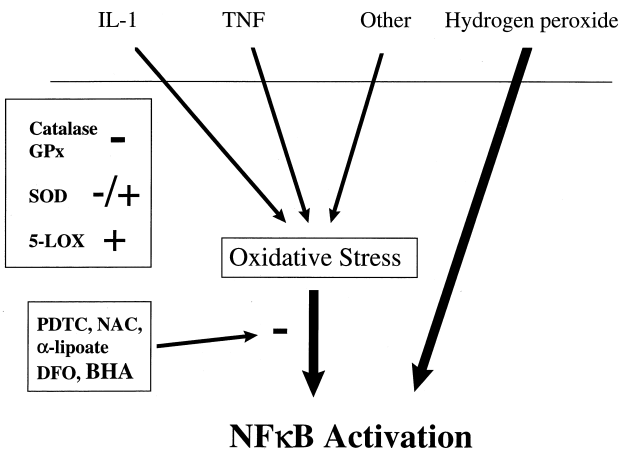

FIG. 2. Summary of the evidence proposed for the oxidative stress model of NFκB activation. This model proposes that diverse NFκB activators all act through a common step involving an increase in oxidative stress within the cell. Evidence is as follows: (1) Hydrogen peroxide can activate NFκB directly in some cells. (2) An increase in intracellular oxidative stress has been measured in response to some stimulants. (3) Antioxidants (PDTC, NAC, α-lipoate, BHA, and DFO) can inhibit pathways to NFκB. (4) Enzymes that modulate the redox status of the cell (catalase, glutathione peroxidases (GPx), SOD, and 5-LOX) can, in some cases, either attenuate (−) or potentiate (+) NFκB activation.

response to agents that also activate NFκB [33–35]. Third, compounds purported to have antioxidant properties such as PDTC can inhibit pathways to NFκB activation [30]. Fourth, inhibition or overexpression of enzymes that affect the level of intracellular ROS has been shown to modulate the activation of NFκB by some agents [34, 36–38]. Ultimately, this theory led to the proposal of H_2O_2 as the central second messenger to NFκB activation [34]. In some ways, this proposal provided a solution to the problem of how diverse agents could activate NFκB: they would all ultimately increase H_2O_2 levels within the cell. However, the identity of a common redox-sensitive or H_2O_2-requiring step has thus far remained elusive.

THE OXIDATIVE STRESS MODEL IN LIGHT OF RECENT FINDINGS

Although many of the upstream and proximal kinases important in NFκB activation have recently been identified, there has been a notable silence as to how the oxidative stress model of NFκB activation can be reconciled with the new data. Recent publications on important pathways to NFκB, such as LPS in monocytes [39] and CD28 in T cells [40], now focus on the role of the IKKs, with no mention of oxidative stress, where previously ROS were discussed as central to these pathways [35, 41, 42]. In particular, no evidence has been presented to substantiate the prediction that the central IκB kinase(s) upon which diverse signalling pathways would converge would be oxidant-responsive or redox-regulated. Additionally, two of the key pathways to NFκB activation, involving IL-1 and TNF, have recently been well characterised from receptor to nucleus (reviewed in [43]), and neither pathway seems to have any obvious requirement for ROS or H_2O_2. Figure 3 illustrates these pathways. Binding of TNF to the TNF receptor type 1 (TNFR1) aggregates three TNFR1s together, inducing the association of TNFR-associated death domain protein (TRADD) with the death domains in the cytosolic region of TNFR1s. TRADD then recruits TNFR-associated factor 2 (TRAF2) and receptor-interacting protein (RIP), both of which are involved in NFκB induction. TRAF2 can then interact with and activate NIK, which can activate the IKKs. On the other hand, binding of IL-1 to its type 1 receptor (IL-1R1) recruits the IL-1 receptor accessory protein (IL-1RAcP), which can activate IL-1 receptor-associated kinase (IRAK) via the adaptor molecule MyD88. IRAK then activates TRAF6 which can associate with TAK1 [25], leading to activation of NIK. Given this substantial increase in our understanding of the components involved in the pathway to NFκB, it is an appropriate time to revisit the evidence for the oxidative stress model of NFκB activation, with a view to re-evaluating it in the light of the recent data.

EARLY WORK IMPLICATING A ROLE FOR OXIDISED THIOLS IN NFκB ACTIVATION

Redox modulation of NFκB activity was first suggested by the Herzenberg group, based on the fact that the NFκB-dependent stimulation of HIV transcription by TNF or PMA was inhibited by NAC, a free radical scavenger and glutathione precursor [44]. They went on to show that in 293 cells, TNF or PMA decreased intracellular thiols and activated HIV long terminal repeat transcription, and that this was inhibited by NAC, which increased thiol levels, or potentiated by diamide, which reduced glutathione levels [45]. They also showed that NAC directly inhibited NFκB activation by TNF and PMA. Therefore, they proposed that NFκB activity was controlled by intracellular thiol levels in that NFκB stimulants somehow led to an increase in oxidative stress, which caused overall thiol levels to be depleted due to a decrease in glutathione levels. In contrast, Toledano and Leonard showed that diamide, a glutathione-oxidising agent, could inhibit NFκB DNA binding *in vitro* [46], an observation that seemed to contradict the previous findings. Further work showed that NFκB was sensitive to oxidative modification of a particular cysteine at position 62 in p50, which is essential for DNA binding [47, 48]. This cysteine is also the point at which thioredoxin, a ubiquitous dithiol-reducing enzyme, acts to stimulate DNA-binding activity of NFκB [48]. Thus, it seemed that *in vitro*, oxidation inhibited while reduction stimulated NFκB-DNA-binding due to the sensitivity of cysteine 62 to oxidation, while *in vivo*, the opposite is true, due to the proposed role of oxidative stress in NFκB activation. Dröge *et al.* attempted to clarify this discrepancy by showing that intermediate levels of oxidised glutathione were required for NFκB activation in lymphocytes, since NFκB activity

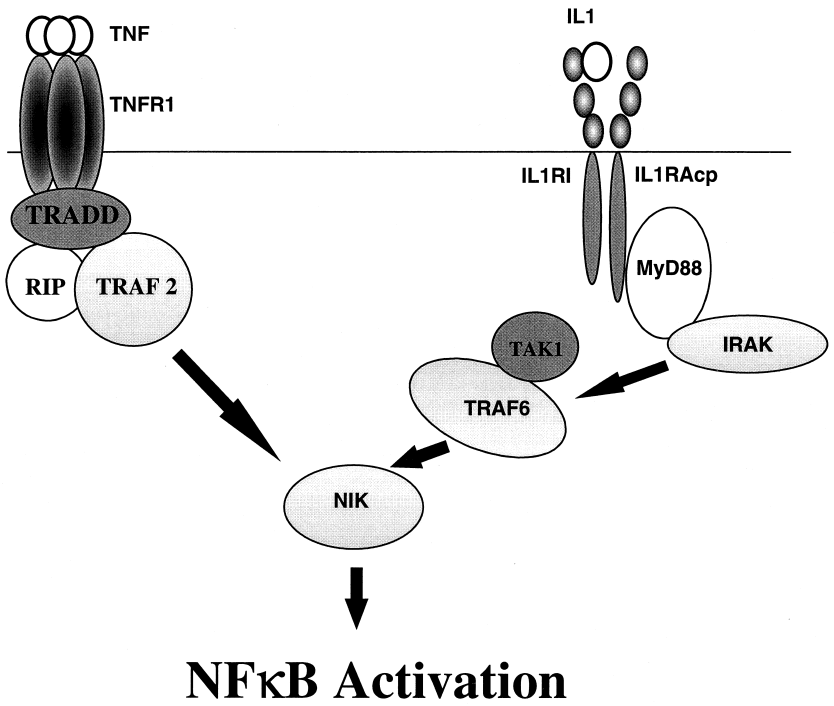

FIG. 3. Pro-inflammatory signal transduction pathways to NFκB. Trimeric TNF binds to and trimerises TNF receptor type 1 molecules (TNFR1), leading to a recruitment of TNFR-associated death domain protein (TRADD), which in turn can recruit TNFR-associated factor 2 (TRAF2) and receptor-interacting protein (RIP), both of which are involved in NFκB induction. TRAF2 activates NIK, leading to IKK activation. Alternatively, IL-1 binds to its type 1 receptor (IL-1R1), leading to recruitment of IL-1R-accessory protein (IL-1RAcp), which activates IL-1R-associated kinase (IRAK) via the adaptor molecule MyD88. IRAK then activates TRAF6, which can associate with TAK1, leading to NIK activation. So far, no direct role for oxidative stress has been demonstrated in either pathway.

may be controlled at two levels by thiols [49]. NFκB activation and nuclear translocation are stimulated by more oxidising conditions, while DNA binding is inhibited by these conditions due to the redox-sensitive cysteine residue. Thioredoxin may protect this residue *in vivo*.

ACTIVATION OF NFκB BY H_2O_2

Schreck *et al.* went a step further and were the first to propose that ROS were actually common second messengers in diverse pathways to NFκB [31]. They based their hypothesis on the fact that direct addition of H_2O_2, itself a source of ROS, to a subclone of Jurkat T cells could activate NFκB. This was prevented by NAC, proposed here to be acting as a free radical scavenger. NAC also blocked the activation of NFκB by cycloheximide, double-stranded RNA, calcium ionophore, TNF, PMA, IL-1, LPS, and lectin. Some of these effects were reported for murine fibroblasts or pre-B cells. Subsequently, H_2O_2 has been shown to activate NFκB in other cell types such as HeLa cells [32]. However, a number of other cell types have proved insensitive to H_2O_2, including monocytic cells [50, 51], astrocytoma [52], standard Jurkats [53] except at high concentrations [54], J. Jhan lymphoblastoid T cells [51], EL4.NOB-1 T cells, KB epidermal cells [54], and human umbilical vein endothelial cells [55, 56]. Thus, activation of NFκB by H_2O_2 may be the exception rather than the rule.

We have reported the potent activation of NFκB by H_2O_2 in ECV304 cells [56], and we used these cells to investigate whether H_2O_2 had a role to play in IL-1- and TNF-mediated NFκB activation. There were a number of lines of evidence to suggest that this was not the case: First, H_2O_2 release from ECV304 was not increased upon stimulation of cells with IL-1 or TNF. Second, pretreatment of cells with H_2O_2 did not potentiate NFκB activation by either cytokine. Third, NFκB activation by H_2O_2 was slow (120 min) and transient compared to the rapid (5 min) and sustained activation seen for the cytokines. Similar slow kinetics for NFκB activation by H_2O_2 and other signals which induce oxidative stress have been reported by other groups. Treatment of a B lymphoma cell line with H_2O_2 required a 2- to 4-hr stimulation [57]. Photosensitisation gave a maximal activation of NFκB at 2 hr compared to a rapid activation stimulated by PMA or TNF in HIV-1-infected lymphocytes and monocytes [58]. Others have shown that NFκB activation by hypochlorous acid in a lymphoid cell line and by H_2O_2 in an epithelial cell line was slower than the response of these cells to cytokines [59, 60]. The much slower activation of NFκB by H_2O_2 may therefore be characteristic of a secondary response of NFκB to agents which induce stress, and may not be relevant to the more rapid cytokine-mediated effects.

There are a number of ways in which H_2O_2 might activate NFκB in a manner distinct from IL-1 and TNF.

H_2O_2 has recently been implicated as having a stimulatory role in a number of systems such as platelet-derived growth factor signal transduction [61], the activation of p42/p44 mitogen-activated protein kinase, and Jun N-terminal kinase [61–63] and in signalling of the small GTP-binding proteins Ras and Rac1 [64, 65]. Thus, it may activate a kinase with the potential to stimulate the IKKs. It is likely that different cell types respond to H_2O_2 in different ways, so that there may be a number of mechanisms by which it might activate NFκB. Sen *et al.* have shown that differences in the kinetics of an increase in intracellular [Ca^{2+}] in response to H_2O_2 may be the basis of the difference in sensitivity of NFκB to H_2O_2 in sensitive and insensitive Jurkat subclones [66]. In another case, H_2O_2 was shown to activate NFκB by inducing TNF release from T-lymphocytic cells [60]. It is likely that H_2O_2 is metabolised by the glutathione redox cycle in many cell types, giving rise to oxidised glutathione, GSSG. Although elevated levels of GSSG inhibit NFκB, Galter *et al.* have suggested that a certain amount of GSSG is required for optimal NFκB activation [67]. Thus H_2O_2-mediated increases in GSSG might in some sensitive cell types activate NFκB. Dröge *et al.* suggested that H_2O_2 could activate NFκB by GSSG-stimulated tyrosine kinase activation [49], and there are reports of H_2O_2-mediated NFκB activation being tyrosine kinase-dependent [57]. H_2O_2- (or GSSG-) induced oxidative damage or modification of a protein or DNA might also be an important trigger in the process. There is evidence that compounds that lead to DNA oxidative damage, such as the anthracycline antibiotic daunorubicin, might initiate a signalling event (distinct from that initiated by cytokines) which activates NFκB [58, 68].

Recently, an alternative pathway to NFκB activation that does not involve S32 and S36 phosphorylation nor IκBα degradation has been elucidated. Hypoxia and reoxygenation have been shown to activate NFκB by this pathway, which involves phosphorylation of tyrosine at position 42 on IκBα and activation of phosphatidylinositol 3-kinase [69, 70]. Interestingly, as well as stimulating tyrosine kinase activity, H_2O_2 can activate phosphatidylinositol 3-kinase [71], suggesting that this pathway might be important for H_2O_2-mediated NFκB activation. Even though H_2O_2 leads to IκBα degradation [56, 59], this might be due to direct oxidation as has been suggested previously [72, 73]. Now that it is possible to measure IKK activity, it will be interesting to see whether H_2O_2 can actually activate IKKα or β. One would have to assume that this has been attempted but found not to be the case.

Hence, although H_2O_2 can activate NFκB, this effect is cell-specific. When activation is seen, the evidence suggests that this is distinct from other activators such as IL-1 and TNF. It remains to be elucidated exactly how H_2O_2 activates NFκB, but it would not be surprising if there were distinct mechanisms of activation in different cell types.

PRODUCTION OF ROS BY NFκB ACTIVATORS

In order for the oxidative stress model of NFκB activation to be valid, stimulants of NFκB must be shown to increase intracellular ROS. This has been achieved in some cases: IL-1 and TNF have been shown to increase ROS in primary human fibroblasts [74], while LPS led to an increase in H_2O_2 production in B-cell lines [30]. H_2O_2 has also been shown to be released in response to agents that activate NFκB in other specific systems [34, 35]. In another study, exhaustive tests failed to show any production of either H_2O_2 or O_2^- in TNF-stimulated L929 cells [75], while Royall *et al.* showed in bovine aortic endothelial cells that TNF had no effect on intra- or extracellular H_2O_2 levels over a period of 12 hr [76]. Others have recently shown that generally, IL-1 is more likely to lead to production of ROS in lymphoid and monocytic cells than in epithelial cells [38, 59]. This all demonstrates that generation of ROS in response to NFκB inducers is not a universal phenomenon, but rather is often a cell- and stimulus-specific phenomenon. As mentioned above, we found that H_2O_2 release from ECV304 was not increased upon stimulation with IL-1, TNF, or PMA at concentrations and times of exposure to stimulants that gave strong activation of NFκB in these cells. We used a sensitive and controlled method to specifically measure H_2O_2, the fluorescent detection of peroxide-mediated scopoletin oxidation [56]. We did find, however, that TNF, but not IL-1, led to a small but significant increase in lipid peroxides, and there is evidence accumulating that some pathways to NFκB may utilise lipid peroxides rather than H_2O_2 (see below).

INHIBITION OF NFκB ACTIVATION BY ANTIOXIDANTS

One of the most compelling lines of evidence for a role for oxidative stress has been the use of antioxidants to inhibit NFκB activation in response to diverse stimuli. Two compounds in particular have been extensively used, NAC and PDTC. Other antioxidants such as vitamin E derivatives [77] and α-lipoic acid [78] have also been used, and shown to inhibit NFκB activation in some cell types. As stated above, NAC is an antioxidant that can increase intracellular levels of glutathione and can also directly scavenge oxidants such as H_2O_2, hydroxyl radical (· OH), and hypochlorous acid (HOCl) [79]. Originally, NAC was shown to inhibit PMA- and TNF-induced activation of the HIV-1 long terminal repeat [44]. Subsequently, it was shown that NAC was acting by blocking NFκB activation by preventing a decrease in intracellular thiol levels [45]. Schreck *et al.* then showed that NAC prevented NFκB activation by diverse stimuli, including H_2O_2, in Jurkat T cells, and concluded that this was due to suppression of ROS [31]. However although NAC has proved inhibitory in some other cells [32], we and others have reported NAC-insensitive pathways to NFκB [52, 54, 56, 59]. Another report [80] showed that in Jurkats and U937 cells,

TABLE 1. Summary of the effect of various putative redox-modulating compounds on pathways to NFκB in ECV304 cells

Compound	Proposed mechanism of action	IL-1	TNF	PMA
NAC	Radical scavenger and glutathione precursor	−	−	−
Allopurinol	Inhibits ROS from xanthine oxidase	−	−	−
DMPO	Hydroxyl radical spin trap	−	−	−
PDTC	Radical scavenger and metal chelator	−	+	+
DDTC	Radical scavenger and metal chelator	−	+	+
DFO	Ferric iron chelator	−	+	+
TEMPO	Ferric iron chelator and ROS scavenger	−	+	+
BHA	Lipid peroxidation	−	+	ND
o-Phenanthroline	Copper chelator	+	+	+
ZM230487	5-LOX inhibitor	+	+	ND
MK886	FLAP inhibitor	−	−	ND
Vitamin C	General antioxidant	+	+	+

Cells were pretreated with physiologically relevant doses of compounds before being stimulated with IL-1, TNF, or PMA for 60 min. NFκB activation was then measured using the electrophoretic mobility shift assay [56]. (−), no inhibition; (+), inhibition; ND, not determined; FLAP, 5-LOX activating protein.

activation of NFκB by the protein phosphatase inhibitors calyculin A and okadaic acid was insensitive to the antioxidants NAC and dihydrolipoate, while in the same cells TNF and PMA were sensitive. Hence, similar to the evidence presented in the previous sections, the effect of antioxidants on NFκB activation has also been shown to be stimulus- and cell-specific.

PDTC seems to be a better general inhibitor of NFκB. As a dithiocarbamate, PDTC has both metal-chelating and antioxidant properties [81]. DDTC has been used for decades in the treatment of metal poisonings, while its metal-chelating properties are also important in its ability to inhibit copper- and zinc-containing superoxide dismutase (CuZnSOD). DDTC has also been shown to retard the onset of AIDS symptoms in some infected individuals [82]. This activity of DDTC has been proposed to be related to the radical-scavenging, antioxidant properties of dithiocarbamates. PDTC is a more stable dithiocarbamate than DDTC [83], which readily penetrates cells and is therefore more commonly used in cell culture. Schreck *et al.* first showed that PDTC inhibited NFκB activation by IL-1, TNF, PMA, LPS, and H_2O_2 in a number of human lymphocytic cells and a mouse fibroblast cell line [33]. They proposed that not only was this strong evidence for a central role of ROS in NFκB activation, but also that PDTC was a specific and universal inhibitor of NFκB that acts independently of the activating agent and cell type used. Others have shown inhibition of NFκB activation by PDTC in different systems [32, 41, 54, 84–86], and the hypothesis that PDTC inhibition of NFκB activation is both specific and universal as well as being strong evidence for a central role of ROS has gained much acceptance. However, like NAC and other antioxidants, inhibition of NFκB by PDTC has now been shown not to be a universal phenomenon [52, 56, 59].

Our results in ECV304 cells using IL-1, TNF and PMA illustrate how even in one cell type, antioxidants and metal chelators can have distinct effects on pathways to NFκB as illustrated in Table 1 [56 and *]. Generally, all three pathways to NFκB were insensitive to NAC and other radical scavengers such as DMPO and allopurinol, while compounds that had antioxidant properties, but also activity against metals, such as PDTC, DFO, and TEMPO, or lipid peroxidation (BHA), inhibited TNF and PMA, but not IL-1. This illustrates the point that PDTC inhibition can be stimulus-specific even within one cell type. Since general antioxidants did not inhibit any of the pathways to NFκB, while metal chelators mimicked the pattern of inhibition seen for PDTC, it also suggested that when PDTC was inhibiting, it was doing so due to its metal-chelating properties. Because DFO, TEMPO, PDTC, and BHA all inhibited activation, we felt that iron-catalysed lipid peroxidation, rather than ROS generation, had a role to play in TNF-mediated NFκB activation in these cells [56]. The role of lipid peroxidation in NFκB activation is discussed more fully in a later section.

Hence, in a particular cell system we found that the effects of PDTC on NFκB were not primarily due to its antioxidant properties. PDTC can also exert a pro-oxidant effect in some cells by increasing oxidised glutathione levels [87, 88], which also leads to an inhibition of NFκB [89], in some cases at least by leading to a chemical modification of NFκB [88]. An interesting approach to the problem of defining a PDTC-sensitive step in a given pathway has been the isolation of mutant cell lines where pathways to NFκB that were sensitive to PDTC in the parent line were inactive, while PDTC-insensitive pathways to NFκB were still functional [90, 91]. In one report, the difference between the parent and mutant line was shown to be independent of the redox status of the cells, and subsequently this group provided evidence that in fact the mutant cell line was lacking the scaffold protein NEMO, a critical component of the IKK-containing signalsome [16]. Upon either transient or stable transfection of the mutant line with NEMO, the PDTC-sensitive pathways to NFκB

* Bowie A and O'Neill LAJ, unpublished results.

were restored, suggesting that in some cell types, NEMO, or an associated protein, may be a target for PDTC. The PDTC-insensitive pathways in these cells included hyperosmotic shock and phosphatase inhibitors, which may have been acting through the alternative tyrosine 42 phosphorylation pathway described above.

We have consistently found that compounds proposed to inhibit NFκB at specific points in a pathway, including antioxidants, often have multiple or unexpected targets on that pathway [88, 92, 93]. Table 1 shows that the copper chelator *o*-phenanthroline inhibited IL-1, TNF, and PMA-stimulated NFκB activation. This was actually due to inhibiting the binding of NFκB to DNA, rather than an upstream effect.* 5-LOX has been proposed as having a role in NFκB activation [35, 38]. We did see inhibition of both IL-1 and TNF using ZM230487, a 5-LOX inhibitor.* However, the 5-LOX activating protein (FLAP) inhibitor MK886 failed to block NFκB even at high doses,* suggesting that ZM230487 may have other targets distinct from 5-LOX. Interestingly, vitamin C, a well-known general antioxidant, inhibited all the pathways to NFκB tested in ECV304.† However, we have shown that this inhibition, at least in the case of TNF, is due to activation of p38 mitogen-activated protein kinase by vitamin C, which then exerts a negative effect on IKK activity.† Another distinct compound that inhibits NFκB, sodium salicylate, has also recently been shown to inhibit the TNF pathway by activating p38 [94], demonstrating a novel negative regulatory role for p38 in IKK activation. Given that in general, TNF has been reported to be more sensitive to redox perturbation than IL-1, it will be interesting to see how many other antioxidants and metal chelators may actually inhibit it by activating kinases such as p38.

Thus, as the pathways to NFκB become more defined, and more potential points of regulation are described, we would predict that many antioxidants purported to inhibit NFκB because of an effect on ROS will actually be shown to have novel targets that may not be due to a direct antioxidant effect. Rather, they may chemically modify proteins or activate kinases or phosphatases that modulate IKK activity. Further, PDTC illustrates the point that a given compound may inhibit NFκB by distinct mechanisms in different cell types. Given our greatly increased knowledge of the NFκB system in recent years, future studies where antioxidants and metal chelators affect NFκB will have to attempt to carefully define the effect of such compounds on a broad range of potential targets for each given pathway and cell type, before assigning a role for oxidative stress in the pathway.

THE EFFECT OF REDOX-MODULATING ENZYMES ON NFκB

A further line of evidence for a role for oxidative stress in NFκB activation has been the effect on NFκB of overexpressing or inhibiting enzymes that modulate the redox state of the cell. In 1992, Schreck *et al.* showed that overexpression of the O_2^--consuming enzyme SOD in MCF-7 cells potentiated TNF-mediated NFκB activation [33]. This led to H_2O_2 itself being implicated as the important oxidative second messenger, since SOD gives rise to it. However, recently contradictory results were found in the same cell line, MCF-7, where overexpression of SOD inhibited multiple signalling pathways induced by TNF, including NFκB [36]. Other work has shown that overexpression of the H_2O_2-degrading enzyme catalase in mouse epidermal JB6 cells inhibited TNF-mediated NFκB activation, while overexpression of SOD potentiated the TNF effect [34]. However, these results are also likely to be cell-specific since another group, using a different cell type (COS-1), could not show that overexpression of catalase affected TNF- (or PMA-) mediated NFκB activation [95].

More recently, a role for glutathione peroxidases, and in particular for phospholipid hydroperoxide glutathione peroxidase (PHGPx), in attenuating some pathways to NFκB activation has been suggested [72]. This would be consistent with the notion presented earlier that lipid peroxides, rather than H_2O_2, may have a role in particular pathways to NFκB, since these enzymes can reduce hydroperoxides such as the products of lipoxygenases such as 5-, 12-, and 15-HPETE (hydroperoxyeicosatetraenoic acid) and peroxidised phospholipids. PHGPx can also indirectly inhibit lipoxygenases, since the latter require a minimum peroxide tone to be catalytically active [72]. We speculated that 5-LOX may be involved in the activation of NFκB by TNF in ECV304 [56], since this enzyme requires DFO-chelatable, redox-active iron, and leads to the generation of lipid peroxides [96]. Interestingly, this enzyme has been implicated in TNF-mediated cytotoxicity [75] and also in NFκB activation by CD28 in primary T cells [35] and by LPS in the promonocytic cell line U937 [42]. Others have recently provided strong evidence that IL-1-mediated NFκB activation in lymphoid, but not epithelial or monocytic cells requires 5-LOX [38, 97]. Indeed, Flohe *et al.* speculate that given the fact that there is obvious interplay between the different ROS-generating and -consuming systems within the cell, much of the evidence for the role of H_2O_2 and ROS in NFκB activation could just as easily be reinterpreted to point to a process such as lipid peroxidation being important [72]. Thus, if a lipoxygenase was shown to be important in particular pathways to NFκB, this might explain some of the effects of oxidants and antioxidants on such a pathway. Perhaps if lipid peroxides are involved, this might implicate 5-LOX products as novel second messengers in some pathways to NFκB. However, it is important to note that in no case has it been shown that activation of 5-LOX (or any other redox-modulating enzyme) alone can

* Bowie A and O'Neill LAJ, unpublished results.
† Bowie A and O'Neill LAJ, manuscript in preparation.

activate NFκB, suggesting that 5-LOX products, or other lipid peroxides, cannot activate NFκB alone; the presence of a physiological stimulant such as TNF is still required.

CONCLUSIONS

Concerning NFκB and oxidative stress we would therefore conclude the following:

1. Many of the important effects, such as activation by H_2O_2 or inhibition by antioxidants, have multiple and complex explanations that are often cell- or stimulus-specific. Hence, these effects do not necessarily point to a central ROS-requiring step in the process. Rather, we would suggest that a central role for H_2O_2 or ROS in a pathway to NFκB is the exception rather than the rule.
2. We hypothesise that many physiological pathways to NFκB do not involve oxidative stress. Some pathways may be redox-modulated, without the need to postulate a central role for oxidative stress, as was originally suggested [51].
3. There is accumulating evidence for a role for lipid peroxides, such as those generated by 5-LOX, rather than H_2O_2 or ROS in some important pathways to NFκB, such as IL-1 in lymphoid cells, although this requirement is cell-specific. Although required in the pathway, their role would be facilitatory rather than causal.
4. It is necessary to treat each pathway to NFκB in a given cell type as distinct, and it is inappropriate to assume that the effects of oxidants and antioxidants on NFκB are cell-independent and always due to redox modulation.

We now have a much clearer understanding of NFκB activation, and recently many of the kinases and adaptor molecules involved in physiological pathways to NFκB have been identified. For those pathways where a role for oxidative stress is likely, the challenge now lies in demonstrating where exactly redox modulation of that pathway occurs.

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