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NF-κB activation by reactive oxygen species: Fifteen years later

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Abbreviations:
Abl, abelson murine leukemia viral
ASK1, apoptosis signal-regulating kinase1
BHA, butylated hydroxyanisole
DMSO, dimethylsulfoxide
GSH, reduced gluthation
JNK, c-Jun N-terminal kinase
MyD88, myeloid differentiation marker 88
NAC, *N*-acetyl-cysteine
PEST, proline, glutamate, serine, threonine
p56Lck, lymphocyte specific tyrosine kinase
PDTC, pyrrolidine-9-dithiocarbamate
PKD, protein kinase D
PKCδ, protein kinase Cδ
ROS, reactive oxygen species
SOD, superoxide dismutase
Syk, spleen tyrosine kinase

ABSTRACT

The transcription factor NF-κB plays a major role in coordinating innate and adaptative immunity, cellular proliferation, apoptosis and development. Since the discovery in 1991 that NF-κB may be activated by H_2O_2, several laboratories have put a considerable effort into dissecting the molecular mechanisms underlying this activation. Whereas early studies revealed an atypical mechanism of activation, leading to IκBα Y42 phosphorylation independently of IκB kinase (IKK), recent findings suggest that H_2O_2 activates NF-κB mainly through the classical IKK-dependent pathway. The molecular mechanisms leading to IKK activation are, however, cell-type specific and will be presented here. In this review, we also describe the effect of other ROS (HOCl and $^1O_2$) and reactive nitrogen species on NF-κB activation. Finally, we critically review the recent data highlighting the role of ROS in NF-κB activation by proinflammatory cytokines (TNF-α and IL-1β) and lipopolysaccharide (LPS), two major components of innate immunity.

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SHIP-1, SH2-containing inositol 5-phosphatase 1
TAK1, transforming growth factor-β-activated kinase
ZAP70, zeta-chain (TCR) associated protein kinase

1. Reactive oxygen species and cellular signalling

Molecular oxygen is an essential molecule for all aerobic life forms, notably for the cell to obtain energy as a form of ATP. Under normal or pathologic conditions, O_2 is often transformed into highly reactive forms, called reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) [1,2]. ROS are generated through multiple sources in the cell, such as the electron transport chain in mitochondria, ionizing radiations [3,4] and through enzymes producing superoxide anion such as phagocytic and non-phagocytic NADPH oxidases [5–7], lipoxygenases [8] and cycloxygenases [9]. Two other oxidant species are physiologically relevant: HOCl produced by the myeloperoxidase from neutrophils [10], and singlet oxygen ($^1O_2$) generated upon photosensitisation and UVA irradiation [11]. Given that ROS are cytotoxic, cells have developed antioxidant defences such as enzymes that dismutate $O_2^{\bullet -}$ into H_2O_2 (SOD-1, -2 and -3) or degrade H_2O_2 (catalase, glutathione peroxidases and peroxiredoxins) [12,13]. When cellular production of ROS overwhelms its antioxidant capacity, a state of oxidative stress is reached leading to serious cellular injuries and contributing to the pathogenesis of several diseases. Nevertheless, if not generated in too high concentration, ROS act as second messengers in signal transduction and gene regulation in a variety of cell types and under several biological conditions such as cytokine, growth factor and hormone treatments, ion transport, transcription, neuromodulation and apoptosis [14,15]. It is now well established that H_2O_2 is the main ROS mediating cellular signalling because of its capacity to inhibit tyrosine phosphatases through oxidation of cysteine residues in their catalytic domain, which in turn activates tyrosine kinases and downstream signalling [16,17]. Depending on the level of ROS, different redox-sensitive transcription factors are activated and coordinate distinct biological responses. A low oxidative stress induces Nrf2, a transcription factor implicated in the transactivation of gene coding for antioxidant enzymes [18]. An intermediate amount of ROS triggers an inflammatory response through the activation of NF-κB and AP-1, and a high level of oxidative stress induces perturbation of the mitochondrial PT pore and disruption of the electron transfer, thereby resulting in apoptosis or necrosis (Fig. 1) [18]. NF-κB was the first transcription factor shown to be redox-regulated [19,20] and this regulation will be the focus of this review.

2. NF-κB and NF-κB-activating pathways

The transcription factor NF-κB is crucial in a series of cellular processes, such as inflammation, immunity, cell proliferation and apoptosis. It consists of homo- or heterodimers of a group of five proteins, namely NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), p65/RelA, c-Rel and RelB [21]. In the resting state, NF-κB is sequestered in the cytoplasm of the cell through its tight association with inhibitory proteins called IκBs, comprising IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p100 and p105. Upon cell stimulation, IκB proteins are rapidly phosphorylated and degraded by the proteasome, and the freed NF-κB

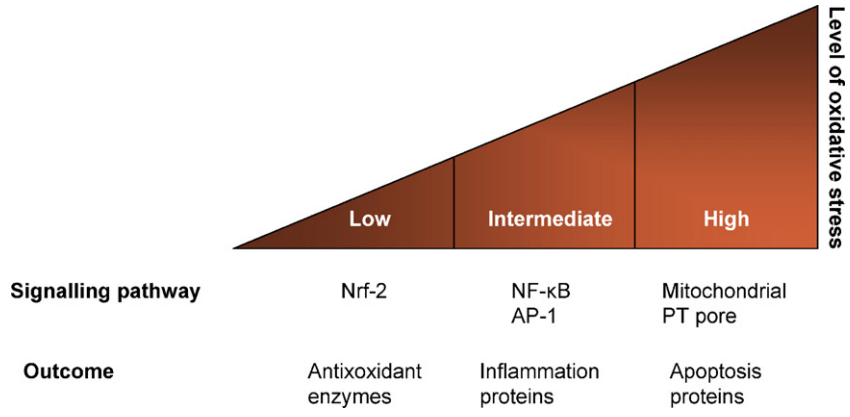

Fig. 1 – Hierarchical oxidative stress model. A low oxidative stress induces Nrf2, a transcription factor implicated in the transactivation of gene coding for antioxidant enzymes. An intermediate amount of ROS triggers an inflammatory response through the activation of NF-κB and AP-1, and a high amount of oxidative stress induces perturbation of the mitochondrial PT pore and disruption of the electron transfer, thereby resulting in apoptosis or necrosis. Adapted from [16].

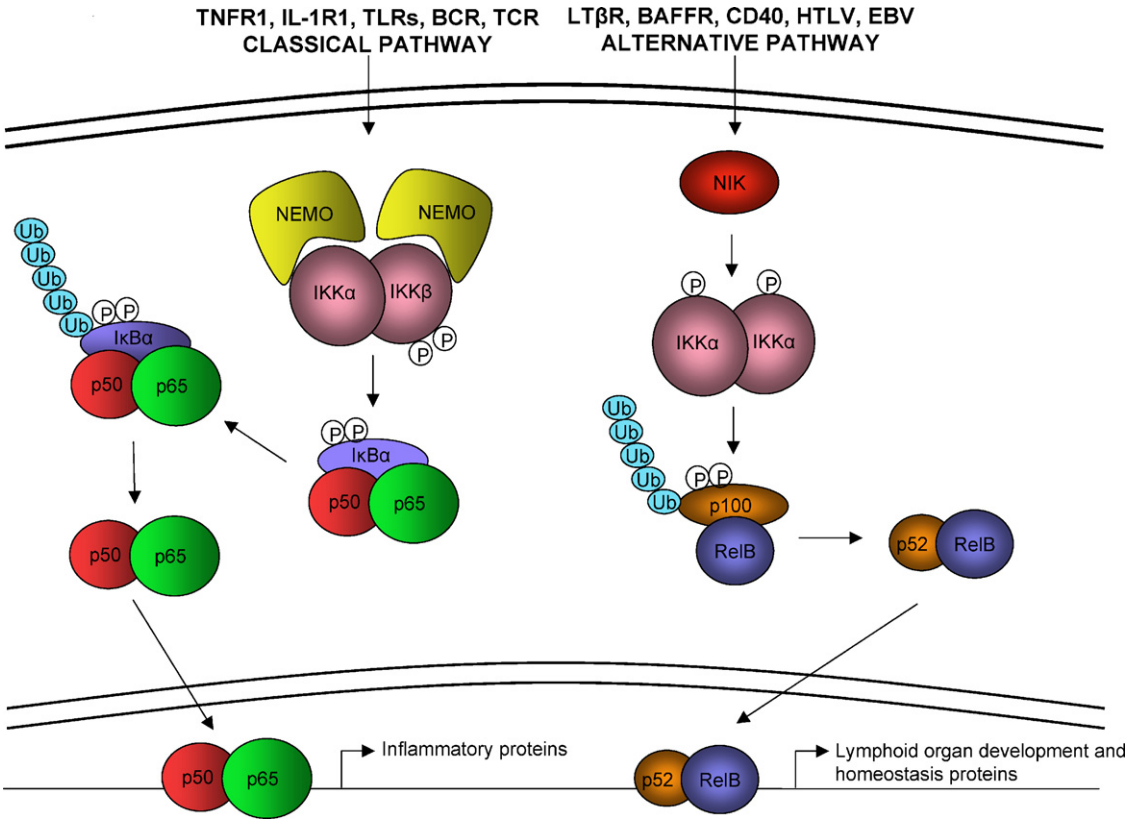

Fig. 2 – Classical and alternative pathways of NF-κB activation. Ligation of TNFR1, IL-1/TLR, TCR and BCR induces IKK-dependent IκBα phosphorylation on S32 and 36, which induces ubiquitination and degradation of the inhibitory protein, thus allowing NF-κB to migrate into the nucleus and transactivate inflammatory genes (classical pathway). Upon ligation of LTβR, BAFFR or CD40 or infection by HTLV or EBV, the alternate pathway is induced. It enhances NF-κB inducing kinase (NIK)- and IKKα-dependent processing of p100 into p52, which binds DNA in association with its partners and stimulates genes implicated in lymphoid organ development and organogenesis. These stimuli also activate the classical pathway.

translocates into the nucleus to regulate the expression of multiple target genes [21]. The sequential events leading to NF-κB activation are now well defined and the current knowledge in this field is briefly summarized below.

2.1. *The classical pathway of NF-κB activation*

The classical NF-κB-activating pathway is induced by a variety of innate and adaptative immunity mediators, such as pro-inflammatory cytokines (TNFα, IL-1β) [22,23], Toll-like receptors (TLRs) [24] and antigen receptors (TCR, BCR) ligation [25–27]. Whereas all these NF-κB inducers signal through different receptors and adaptor proteins, they all converge to the activation of the so called IκB-kinase (IKK) complex, which includes the scaffold protein NF-κB essential modulator (NEMO, also called IKKγ) [28], IKKα and IKKβ kinases [29]. Once activated by phosphorylation, the IKK complex phosphorylates IκBα on Ser32 and Ser36, which is subsequently ubiquitinated and degraded via the proteasome pathway. The freed NF-κB then translocates into the nucleus where it activates the transcription of target genes such as cytokines, chemokines, adhesion molecules and inhibitors of apoptosis (Fig. 2) [26].

2.2. *The alternative pathway of NF-κB activation*

Beside this classical activation, a novel NEMO-independent NF-κB-activating pathway, important for secondary lymphoid organ development and homeostasis and adaptive immunity, was described. It is induced by B-cell activating factor (BAFF) [30], lymphotoxin β (LTβ) [31], CD40 ligand [32] and human T cell leukemia (HTLV) and Epstein-Barr (EBV) virus [33,34]. It enhances NF-κB inducing kinase (NIK)- and IKKα-dependent processing of p100 into p52, which binds DNA in association with its partners, like RelB. These stimuli also activate the classical pathway (Fig. 2).

3. NF-κB activation by H_2O_2

The vast majority of studies concerning oxidant-induced NF-κB activation have used H_2O_2 as a direct source of ROS. After its production in the mitochondria or through specialised enzymes, superoxide anion ($O_2^{\bullet -}$) is rapidly metabolized into H_2O_2 via the following dismutation reaction: $2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$. This reaction occurs either spontaneously or is catalysed in cells by superoxide dismutase

(SOD). H_2O_2 is a mild oxidant mediating its effects by itself or via its transformation into $OH^{\bullet}$ in the presence of Fe^{2+} through the so-called Fenton reaction [35]. Whether it is H_2O_2 or $OH^{\bullet}$ that mediates NF-κB activation is still a matter of debate since their relative steady-state concentrations strongly depend on cellular antioxidant defences and metal content.

In 1991, Schreck et al. were the first to demonstrate that direct addition of H_2O_2 to the culture medium of a subclone of Jurkat cells (Jurkat JR) could activate NF-κB [19]. Since this discovery, several laboratories have put considerable efforts into dissecting the molecular mechanisms underlying this activation. The results that came out of these studies suggest that NF-κB activation by H_2O_2 is highly cell-type specific and involves quite different mechanisms [36]. Here, we will describe the current knowledge in that matter.

3.1. H_2O_2-induced NF-κB activation in T cells: the IKK-dependent pathway comes back into fashion

Oxidant-induced signalling pathways have been intensely subjected in T cell lines for many reasons. First, T cells are often submitted to ROS during inflammatory response, which can, in turn, influence a number of signalling pathways. For example, at a site of inflammation, H_2O_2 is produced by activated macrophages and neutrophils at an estimated rate of $2–6 \times 10^{-4}$ μM/h per cell and T cells may be exposed to 10–100 μM H_2O_2 in a physiological environment [15]. Secondly, it is now clear that the activation of T cells through their antigen receptors increases the level of intracellular ROS that, instead of being toxic, can actually play a positive role in controlling signalling pathways that lead to T cell proliferation [37]. Thirdly, T cell apoptosis is clearly regulated by ROS [38]. For example, a recent study in Jurkat leukemic cells has shown that NF-κB activation by H_2O_2 induces Bfl-1, which, in turn, attenuates Fas-mediated apoptosis [39]. Moreover, some compounds used in anti-leukemic chemotherapies induce cell death through ROS generation [40,41]. For all these reasons, understanding NF-κB activation mechanism by ROS in T cell was of importance. Until recently, all the works concerning NF-κB activation by ROS in T cells have highlighted an atypical mechanism of activation totally distinct from those triggered by pro-inflammatory cytokines. It involves phosphorylation of the inhibitor IκBα on tyrosine 42 rather than the classical ser 32 and 36 by the IKK complex. This was true in murine T lymphocytes [42] and in human Jurkat T cells [43,44]. Furthermore, the IκBα degradation mechanism appears to be proteasome-independent, but instead relies on a calpain-mediated digestion after phophorylation on S/T in the so-called PEST sequence of the inhibitor [42]. NF-κB activation induced by tyrosine phosphorylation of IκBα was also observed after pervanadate (a potent tyrosine phosphatase inhibitor) and hypoxia/reoxygenation treatment [44,45]. This can occur in the absence of IκBα degradation; in this case, a dissociation from NF-κB has been described [46]. The discovery of the terminal tyrosine kinase that phosphorylates IκBα Ytyr42 has been a challenge for many years. Livolsi et al. first demonstrated that the TCR-associated tyrosine kinases p56Lck and ZAP-70 were required for pervanadate-induced IκBα tyrosine phosphorylation, without showing that these kinases indeed phosphorylate IκBα directly [44]. Recently, Takada et al. reported that Syk tyrosine kinase was required for H_2O_2-induced IκBα tyrosine phosphorylation and NF-κB activation, and was capable of phosphorylating IκBα in vitro, suggesting that Syk may be the terminal tyrosine kinase responsible for IκBα tyrosine phosphorylation [43]. Our group has recently called this ''Y42 paradigm'' into question by studying the H_2O_2-induced NF-κB activation mechanism in T cells other than Jurkat cells, namely CEM and Jurkat JR (also termed Wurzburg). Unexpectedly, micromolar amounts of H_2O_2 were shown indeed capable of inducing IKK activation in these cell lines, leading to a classical IκBα phosphorylation on Ser32 and 36 [47]. No tyrosine phosphorylation was observed in this case. However, pervanadate treatment still induced a strong tyrosine phosphorylation of IκBα, suggesting that NF-κB activation mechanisms by H_2O_2 and pervanadate are different, at least in CEM and Jurkat JR cells [47]. In fact, the differences between Jurkat versus CEM and Jurkat JR cells in term of oxidant-induced NF-κB activation mechanism relied on the expression of the SHIP-1 protein. SHIP-1, a lipid phosphatase, acts by dephosphorylating the membrane-bound PtdIns(3,4,5)P_3, generated by PI3Kinase, and has thus been described as a negative regulator of immune receptor, cytokine and growth factor receptor signalling [48]. Furthermore, SHIP-1 can interact with a large number of proteins via its SH2- and NPXY-containing domains, thus influencing numerous signalling pathways [48]. It is now well known that Jurkat cells are deficient in SHIP-1 expression at the protein level, but that CEM cells express the protein normally [47,49], which can, in turn, influence a number of signalling pathways [50]. The rescuing of Jurkat cells with SHIP-1 clearly made them shift to a classical mechanism dependent on IKK activation and phosphorylation of IκBα on ser 32 and 36 upon H_2O_2 stimulation. Furthermore, a less pronounced tyrosine phosphorylation of IκBα was observed in this case (Fig. 3) [47]. As mentioned above, this observation was also made in Jurkat JR cells which are more sensitive to oxidant-induced NF-κB activation than the parental cell line Jurkat [19,51], and express SHIP-1 normally. The analysis of the NF-κB activation pathway upon oxidative stress treatment in that cell-type also revealed an IKK-dependent mechanism [47]. This observation could explain why NF-κB activation might be more rapid and important in that subclone than in Jurkat cells, as observed by several authors [51,52]. All these data clearly suggest that the atypical NF-κB activation pathway described in Jurkat cells treated by oxidative stress is only available in that cell type. NF-κB activation in other T cell lines is the classical IKK-dependent mechanism that relies on SHIP-1 (Fig. 3). The tyrosine-phosphorylation mechanism is probably a rescue pathway adopted by SHIP-1 negative cells. The exact mechanism by which SHIP-1 acts to activate the IKK complex has still to be delineated. Both phosphatase and SH2 domains of SHIP-1 seem to be crucial in this process, but considerable work has yet to be carried out to find out the exact role of that protein in NF-κB redox regulation.

3.2. H_2O_2-induced NF-κB activation in epithelial cells: the crucial role of PKD in IKK activation

The signalling pathway leading to NF-κB activation by H_2O_2 in HeLa cells was recently delineated by Toker's laboratory [53,54].

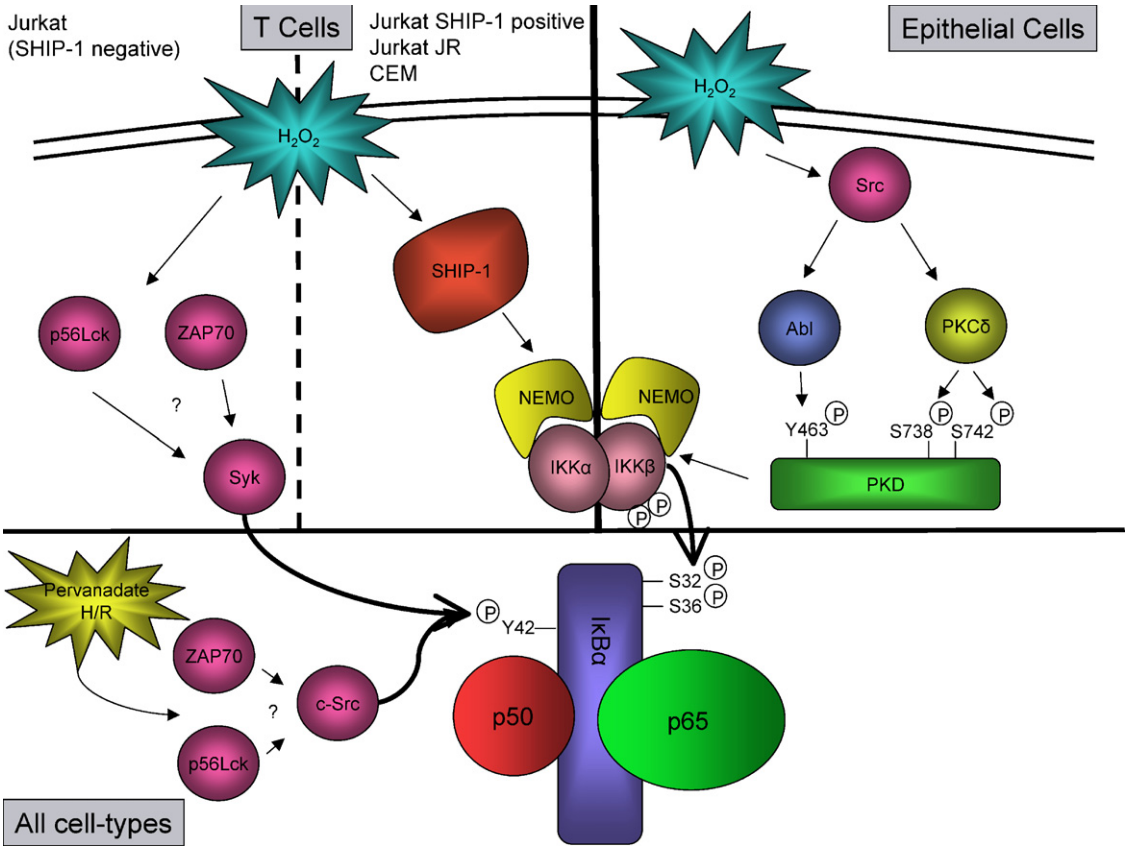

Fig. 3 – Mechanisms of H_2O_2-induced NF-κB activation in T cells and epithelial cells. In T cells, H_2O_2 induces a Syk-mediated tyr 42 or an IKK-induced ser 32 and 36 phosphorylation of IκBα, depending on the expression of the inositol phosphatase SHIP-1. In epithelial cells, H_2O_2 triggers IKK complex activation through PKD activation. Pervanadate induces IκBα tyrosine phosphorylation in all studied cell-types in a c-Src-dependent fashion. H/R: hypoxia/reoxygenation. See text for details.

They showed that H_2O_2 induces IKKβ activation and NF-κB transcriptional activity via activation of PKD. ROS activate PKD by two Src-mediated signalling pathways. First, activated Src induces Abl-mediated phophorylation of PKD at Y463 in the PH domain. This facilitates release of the PH domain, which exposes the catalytic domain and activation loop residues to a second phosphorylation by PKCδ on S738/S742. This induces a fully activated PKD which, in turn, activates the IKK complex (Fig. 3). They also showed that this signalling pathway mediates cellular survival in response to ROS, which reinforces the crucial role of NF-κB activation in protecting cells from ROS-induced apoptosis [53,54]. It should be noted that tyrosine phosphorylation of IκBα has also been reported in HeLa cells treated by pervanadate and hypoxia/reoxygenation, suggesting that both mechanisms of activation may coexist in that cell-type, depending on the nature of oxidative stress. In that case, the tyrosine kinase c-Src has been reported to be responsible for IκBα tyrosine phosphorylation (Fig. 3) [55].

4. NF-κB inhibition by ROS: the case of lung epithelial cells

Very few works have highlighted an inhibitory effect of H_2O_2 on NF-κB activation by pro-inflammatory cytokines. Nevertheless, a simultaneous exposure to pro-inflammatory mediators and ROS is likely to occur in inflammatory states. Korn et al. reported that, in this case, H_2O_2 is capable of inhibiting TNF-induced NF-κB activation in lung epithelial cells by reducing IKKβ activity through oxidation of cysteine residues in the IKK complex [56]. One likely candidate is cysteine 179 in the IKKβ kinase domain. Furthermore, other studies have shown that cyclopentenone prostaglandins and arsenite, which are potent NF-κB inhibitors, target the IKKβ cysteine 179 [57,58]. Modification of this amino acid might inactivate IKK complex by altering its conformation. Finally, NF-κB inhibition was also observed by ROS induced by cigarette smoke condensate in alveolar epithelial cells. Altogether, these data suggest that ROS may have an inhibitory effect on NF-κB activation in lung epithelial cells, on the contrary of other cell types [59].

5. Modulation of NF-κB activation by other reactive oxygen species and reactive nitrogen species

Although the vast majority of studies concerning oxidant-induced NF-κB activation have focussed on H_2O_2, other oxidants, like hypochlorous acid (HOCl) and singlet oxygen

($^1O_2$), have been shown to modulate NF-κB activation. On the other hand, some works have also highlighted NF-κB regulation by peroxinitrite which is a reactive nitrogen species. In this chapter, we will briefly summarize the current knowledge in that matter.

5.1. Modulation of NF-κB activation by HOCl

During phagocytosis of bacteria, neutrophils produce hypochlorous acid (HOCl) into phagolysosomes or into the extracellular medium. HOCl is formed from H_2O_2 and Cl^- ion by myeloperoxidase [10]. HOCl is a strong oxidant that kills phagocytosed bacteria, but can also react with amines to produce chloramines and *N*-chlorinated derivatives which have long lifetimes [60]. These chloramines retain the oxidizing capacities of HOCl and are also playing a protecting effect on the surrounding cells. Among these chloramines, taurine chloramine (TauCl) is generated in great amount in HOCl-producing neutrophils because these cells contain high concentrations of taurine, a free amino acid not incorporated in proteins. Although a pioneer work suggested that HOCl can activate NF-κB in lymphocytic cells [61], following studies revealed that HOCl-derived chloramines are potent NF-κB inhibitors. For example, TauCl was shown to decrease LPS-induced NF-κB activation and IKK activity in alveolar macrophages, resulting in inhibition of iNOS and TNFα gene expression [62]. The molecular mechanism of this inhibition relied on oxidation of IκBα methionine 45, which renders the inhibitor resistant to TNF-induced degradation [63]. Other works demonstrated that ammonia monochloramine (NH_2Cl) and glycine chloramine (GlyCl), two others neutrophils-derived oxidants, but not TauCl, were capable of inhibiting TNF-induced NF-κB activation via the same molecular mechanism [64]. This apparent discrepancy is explainable by the fact that TauCl is membrane-impermeable, whereas NH_2Cl and GlyCl are membrane permeable, and are thus capable of regulating redox signalling pathways more efficiently and at much lower concentrations than TauCl [65,66]. The results obtained by Kanayama et al., describing an inhibitory effect of TauCl on NF-κB activation, are explainable by the fact that they added TauCl to cells in culture medium that contains other amino acids, whereas others authors used amino acid-free solutions. Since chloramines can undergo transchlorination reactions with other amines in the medium, they likely transformed in more permeable chloramines like GlyCl [67]. Altogether, these results demonstrate that, on the contrary of H_2O_2, HOCl and its derivatives are apparently strong inhibitors of the NF-κB pathway, which can result in a diminution of the inflammatory response in HOCl-producing cells.

5.2. NF-κB activation by $^1O_2$

Singlet oxygen ($^1O_2$) is a highly oxidative species produced by energy transfer upon photosensitisation and UVA irradiation. $^1O_2$ was shown to induce NF-κB activation in pyropheophorbide-a methylester-mediated photosensitisation of endothelial cells [11] and UVA irradiation of human skin fibroblasts [68]. The detailed mechanisms of this activation will not be presented here. We encourage interested people to read two recently published reviews on that topic [69,70].

5.3. Modulation of NF-κB activation by peroxinitrite

Peroxinitrite ($ONOO^-$, PN) is formed by the reaction of nitric oxide (NO) with superoxide ($O_2^{\bullet -}$) [71], and is thus generated when the production of NO and $O_2^{\bullet -}$ is enhanced, notably under inflammatory conditions, circulatory shock and reperfusion injury [72]. PN formation also occurs in the heart during myocardial infraction [73] and heart failure [74]. PN is a highly oxidant and nitrating species causing important cellular injuries and is associated with numerous pathologies. Recently, PN was also demonstrated to modify redox-sensitive cellular signalling pathways, such as the NF-κB pathway. PN was shown by several authors to activate NF-κB in endothelial cells [75], leukocytes [76] and vascular smooth muscle cells [77]. However, a recent work carried out by Levrand et al. clearly demonstrated that PN is a potent inhibitor of NF-κB activation triggered by inflammatory stimuli in cardiac and endothelial cell lines [78]. They demonstrated that PN blocks IKKβ phosphorylation and activation, thereby preventing NF-κB nuclear translocation. The PN inhibitory effect on NF-κB activation was further confirmed by Park et al. [79]. They showed that PN is capable of inducing p65 tyrosine nitration on Y66 and Y152, which, in turns triggers replacement of p65/p50 dimers with the repressive p50/p50 complex on promoters and subsequent association of p65 with IκBα to promote export [79]. Therefore, the data collected about the effects of PN on the NF-κB pathway are apparently contradictory and still a matter of debate [80].

6. Involvement of reactive oxygen species in NF-κB activation by pro-inflammatory cytokines and LPS

To explain the fact that such a diversity of inducers activate NF-κB via the same IKK-dependent pathway, a model has emerged suggesting that all NF-κB activators cause an oxidative stress that is mainly responsible for IKK activation and IκBα degradation. This model is based on several observations, including that most of NF-κB-inducers trigger the formation of ROS [81,82] and that several antioxidants can block NF-κB activation [83]. Indeed, an important number of papers have been published concerning the effects of different antioxidants (NAC, PDTC and GSH) or over-expression of antioxidant enzymes (SOD) on NF-κB activation by TNFα and IL-1β. These results have been well summarised in recent reviews [84,85] and will not be presented here. Furthermore, this research area is still a matter of intense debate, mainly because of many conflicting reports [36,86–89]. These discrepancies may be explained by the fact that antioxidants have broad effects on cellular physiology and the use of such compounds can result in artefactual results. Therefore, in this section, we will only focus on recent and unambiguous molecular data highlighting the role of ROS in cytokine-induced NF-κB activation.

6.1. Involvement of ROS in NF-κB activation by IL-1β

IL-1β is a potent pro-inflammatory cytokine that exerts its effects by binding to its receptor (IL1-R1) on the plasma

membrane. This binding induces the recruitment to the receptor cytoplasmic tail of adaptator and effector proteins, including IL-1RacP, MyD88 and Tollip [90–92]. MyD88 then mediates the recruitment of the interleukin-1 receptor-associated kinase (IRAK) family members to the IL-1R [93], which, in turn, recruit TRAF6 [94]. Then, TRAF6 recruits TAK1 that mediates phosphorylation of the IKK complex, a crucial step in NF-κB activation [95]. The redox dependence of NF-κB activation by IL-1β was first shown by Bonizzi et al. to be cell-type specific. These authors showed that ROS production was required for NF-κB activation by IL-1β in both lymphoid and monocytic cells, but not in epithelial cells [82]. Using specific inhibitors, they identified 5-lipoxygenase (5-LOX) as the main source of ROS after IL-1β induction in lymphoid cells. 5-LOX is the first enzyme of the leukotriene biosynthesis pathway; it catalyses the insertion of molecular oxygen on C-5 of arachidonic acid. In monocytic cells, the main source of ROS in IL-1β-induced NF-κB activation was shown to be the NADPH oxidase complex [96]. Although at that time no ROS-dependence in NF-κB activation by IL-1β was demonstrated in epithelial cells, an elegant recent study, carried out by Li et al., called this idea into question. They showed that in MCF7 epithelial cells, IL-1β stimulation induces MyD88-dependent endocytosis of IL-1R1, and that this event is required for the redox-dependent NF-κB activation. During this endocytosis, Nox2 (a phagocytic NADPH oxidase also expressed in non-phagocytic cells) is recruited to the endosomal compartment in a Rac1-dependent fashion. Rac1, a small GTPase, plays a key role in activating $O_2^{\bullet-}$ production by NADPH oxidases. $O_2^{\bullet-}$ is thus produced in the IL-R1/Nox2-containing endosomal compartment. $O_2^{\bullet-}$ spontaneously dismutates into H_2O_2, which diffuses outside the endosome. This local oxidative stress triggers a TRAF6 association with the receptor complex on the ligand-activated endosome, which leads to IKK and NF-κB activation (Fig. 4) [97]. The same authors have also reported that H_2O_2-mediated regulation of NIK is important in IL-1β-mediated induction of NF-κB [98]. Since the role of NIK in NF-κB activation by classical inducers (such as TNFα or IL-1β) is very controversial ($NIK^{-/-}$ Mefs display unimpaired signalling in response to TNFα or IL-1β in terms of NF-κB activation [99]), these results must be interpreted cautiously.

6.2. *Involvement of ROS in NF-κB activation by TNFα*

Like IL-1β, TNFα is a potent pro-inflammatory cytokine that plays a crucial role in a series of cellular events such as apoptosis, cell proliferation, differentiation and septic shock [100]. It binds to its cellular TNFR1 receptor, which triggers signalling cascades that activate NF-κB and AP-1 transcription factors. The signalling pathway that leads to NF-κB activation is now well established [21,101]. The ligation of TNFR1 by trimeric TNFα leads to the aggregation of the receptor and dissociation of silencer of death domain (SODD), an inhibitor of TNFR1 activity, which allows binding of TNFR-associated death domain protein (TRADD protein) [102]. TRADD subsequently recruits downstream adapters like TNF-receptor-associated factor (TRAF proteins) [103]. Although many members of the TRAF family have been implicated in TNF signalling, it appears that both TRAF2 and TRAF5 have a role in NF-κB activation by TNFα [104]. Receptor interacting protein 1

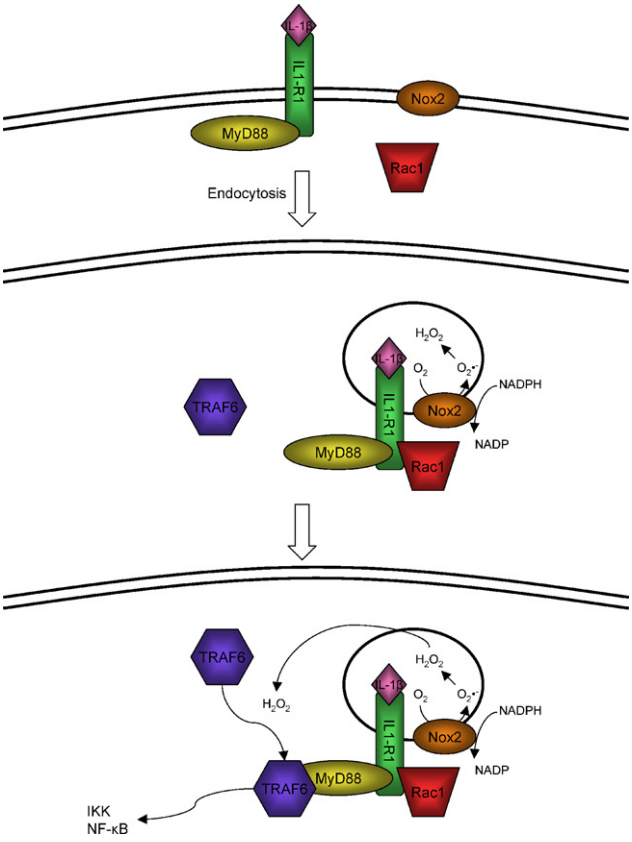

Fig. 4 – NF-κB activation by IL-1R1 depends on receptor endocytosis and endosomal ROS formation. IL-1β stimulation induces MyD88-dependent endocytosis of IL-1R1, an event required for NF-κB activation through Nox2-dependent H_2O_2 production. See text for details. Adapted from [97].

(RIP1) also plays a crucial role in NF-κB activation by TNFα [105]. RIP1 functions as a scaffold protein notably through its direct binding to NEMO, which allows the recruitment of the IKK complex in TNF signalling [106].

As mentioned above, antioxidants have been reported to inhibit TNF-induced NF-κB activation [19,51,83,107], but the molecular mechanisms underlying this observation are, contrary to IL-1β signalling, still poorly understood and were furthermore recently called into question by Hayakawa et al. [87]. They showed that, whereas NAC and PDTC efficiently blocked TNF-induced IκBα degradation and NF-κB activation, the more potent antioxidants epigallocatechin-gallate (EGCG) and Vitamin E analog Trolox failed to inhibit TNF-stimulated NF-κB activation, suggesting that the effect of NAC and PDTC on NF-κB signalling does not rely on their antioxidant capacities, but rather acts by inhibiting a crucial step in TNF signalling. Indeed, they showed that NAC inhibits TNF-stimulated signal transduction by lowering the TNF receptor affinity, and that PDTC is likely to inhibit IκB-ubiquitin ligase activity. These results are reinforced by the observation that, whereas NAC does not inhibit IL-1 or TPA-induced IκBα degradation, PDTC does, suggesting that NAC acts specifically on the early events in TNF signalling, but that PDTC has a larger effect by inhibiting IκBα degradation induced by a broad

range of inducers. Finally, they showed that TNF-induced production of ROS only appears after 2 h of TNF treatment, which does not explain the NF-κB activation which already takes place after 10 min.

Acetylation and deacetylation events are also implicated in the regulation of NF-κB transcriptional activity upon TNF-induction, which, in turn, can modify the inflammatory response [108]. The effects of ROS on the modulation of histone acetyltransferases (HAT) and deacetylases (HDAC), the key enzymes responsible for chromatin remodelling, are still poorly understood. The hypothesis that oxidants may play a role in the modulation of HDAC has been recently proposed by Ito et al. and Moodie et al. [59,109]. They showed that ROS (induced by cigarette smoke or H_2O_2 treatment) reduce HDAC2 expression and activity and increase acetylation of histone H4 in alveolar epithelial cells, which could in turn modify gene transcription and augment inflammatory response, especially in the case of cigarette-induced chronic obstructive pulmonary disease. The readers can obtain more information about that research area in a recent review by Rahman et al. [110]. Finally, it should also be noted that NAC was shown to inhibit p65 ser536 phosphorylation, suggesting that post-translational modifications affecting p65 are also redox-sensitive [111].

6.3. *ROS-mediated crosstalk between NF-κB and JNK upon TNFα stimulation*

Whereas the relevance of ROS in TNF-induced NF-κB activation is still controversial, their importance in mediating the cross-talk between JNK and NF-κB activation upon TNF induction is now well characterized. As mentioned above, TNFR1 ligation triggers activation of both NF-κB and JNK signalling, two pathways having opposite biological roles. Even if that research area is still a matter of controversy, one can say by and large that JNK activation promotes apoptosis via the mitochondrial-dependent pathway [112], whereas NF-κB activation promotes cell survival by upregulating the expression of antiapoptotic members of the Bcl2 family and caspase inhibitors [113]. It has also been reported that NF-κB can inhibit apoptosis by down-regulating JNK activation. Tang et al. and De Smaele et al. have demonstrated that TNF induces prolonged JNK activation in NF-κB activation-deficient cells (p65/RelA and IKKβ knockouts and cell expressing degradation-resistant IκBα), which, in turn, promotes apoptosis, suggesting that TNF-induced NF-κB target genes block JNK activation [114,115]. In that respect, they identified growth arrest and DNA damage-inducing protein 45β (GADD45β) and X chromosome-linked IAP (XIAP) as capable of inhibiting JNK signalling by inactivating MEKK7 (which triggers the JNK pathways) [116] and inhibiting caspase activation, respectively. However, analysis of *Gadd45β*$^{-/-}$ and *Xiap*$^{-/-}$ fibroblasts failed to reveal changes in the kinetics of JNK activation, making the molecular mechanism by which NF-κB down-regulates JNK quite controversial [117,118]. Recently, this mechanism was more deeply delineated and the crucial role of ROS has emerged. In fact, several laboratories have independently reported that NF-κB down-regulates JNK activation by suppressing TNF-induced ROS accumulation [119–122] (reviewed in references [123,124]). They showed that TNF-induced ROS production is responsible for sustained JNK activation in NF-κB-activation deficient cells, whereas wild-type cells exhibited neither ROS production nor sustained JNK activation upon TNF challenge. Moreover, prolonged JNK activation is inhibited by pre-treatment of NF-κB-defective cells with the antioxidants BHA or NAC, suggesting that ROS are key messengers of prolonged JNK activation after TNF induction. The molecular mechanism by which ROS activate JNK has been recently reported [122]. Indeed, ROS inactivate MAP kinase phosphatases (MKPs, which are known to suppress JNK activation) by oxidizing critical residues in their phosphatase domain, which leads to prolonged JNK activation (Fig. 5). Moreover, oxidized MKPs are rapidly degraded by the ubiquitin-proteasome pathway. Several data may explain how NF-κB, when present, inhibits TNF-induced ROS accumulation. For example, a number of antioxidant enzymes like MnSOD were reported to be expressed in response to TNF in an NF-κB-dependent fashion [125], which can explain a more efficient ROS clearance after TNF induction in wild-type cells, and a sustained ROS production in NF-κB-defective cells.

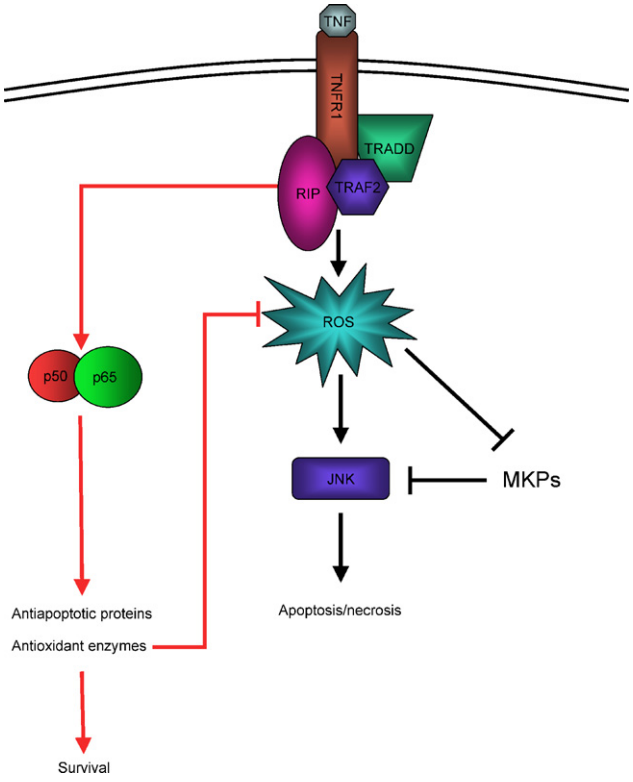

Fig. 5 – ROS-mediated crosstalk between NF-κB and JNK. In NF-κB deficient cells,TNF induces ROS production that inactivate MKPs, which in turn allows sustained JNK activation and apoptosis (black arrows). When present, NF-κB induces ROS clearance by transactivating genes coding for antioxidants enzymes, which inhibit JNK activation and enhance cellular survival (red arrows).

6.4. *Involvement of ROS in NF-κB activation by LPS*

LPS is an endotoxin found in the outer membrane of Gram-negative bacteria. It activates host innate immunity by stimulating phagocytic cells (monocytes/macrophages and

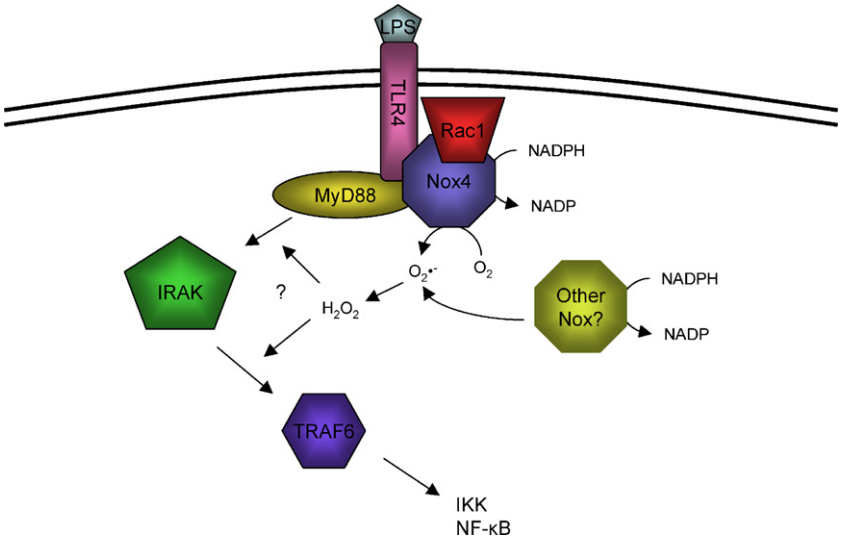

Fig. 6 – NF-κB activation by TLR4 requires Nox4-dependent ROS formation. LPS-induced ROS generation and NF-κB activation are mediated in part by direct interaction of TLR4 with Nox4. The possible role of another Nox enzyme is indicated. See text for details.

neutrophils) to produce proinflammatory cytokines like IL-1, IL-6 and TNF-α [126]. LPS is recognized by TLR4, a member of the TLR family that is involved in innate immunity and inflammation response. Upon binding of TLR4 to LPS, the cytoplasmic region of TLR4 recruits MyD88, which links TLR4 to IRAK and TRAF6 that mediates NF-κB activation [127,128]. CD14, which is expressed on the surface and in the cytoplasm (sCD14) of monocytes/macrophages and neutrophils, has also been reported to play a key role in the recognition of LPS and in downstream cytokine release, notably through its interaction with LPS-binding protein (LBP), which binds the lipid A region of LPS and aids LPS to dock at the TLR4 [129–131]. Involvement of ROS in NF-κB activation by TLR4 has been suggested using antioxidants. Pre-treatment of neutrophils with NAC or α-tocopherol prevented LPS-induced NF-κB activation and the production of pro-inflammatory cytokines [132], and NAC and DMSO were reported to block NF-κB activation and IL-8 secretion in monocyte-like THP-1 cells challenged with LPS [133]. Sanlioglu et al. [134] went one step further demonstrating for the first time that the activation of Rac1 and the subsequent production of ROS are key steps involved in NF-κB activation and TNF secretion in macrophages challenged with LPS. Using blocking antibodies, they also reported that the ROS-dependent Rac1 activation is independent of the CD14 receptor, suggesting that alternative pathways contribute to NF-κB activation by LPS [134]. The exact molecular source of ROS upon LPS challenge was recently discovered by Park et al. [135]. They showed that, in HEK293T cells, LPS-induced ROS generation and NF-κB activation are mediated by direct interaction of TLR4 with NADPH oxidase 4 (Nox4), a protein related to the phagocytic cells NADPH oxidase 2 (Nox2), but only confirmed partially this role in the U937 monocytic cell line, suggesting that another Nox enzyme might be involved in LPS-induced NF-κB activation in U937 cells (Fig. 6) [135]. Whether this local oxidative stress triggers activation of TRAF6, as with the IL-1β signalling pathway, is currently unknown. It should, however, be noted that ROS production after LPS challenge has been showed to mediate the formation of a complex between TRAF6 and the redox-sensitive ASK1, which, in turn, triggers p38 activation, another downstream target of LPS signalling [136].

7. Conclusions and perspectives

NF-κB redox regulation has been intensely studied in several cell-types and biological conditions. It is now clear that H_2O_2-induced NF-κB activation mechanism relies mainly on IKK activation, but the redox-sensitive pathways triggering this activation are quite different depending on the cell-type considered, which renders the drawing up of consensual models and the establishment of therapeutical strategies quite difficult to consider. The solution would be to study NF-κB redox regulation in primary cells, a choice unfortunately too rarely taken mainly due to lack of reproducibility. Different antioxidant status between donors may explain such discrepancies, but this can also be the consequence of artefactual results obtained with cultured cell lines [137]. It will be a challenge in the future to overcome these difficulties and study NF-κB redox regulation in more relevant systems. Over the past 15 years, the role of ROS in NF-κB activation by inflammatory cytokines and LPS has also been the subject of intense studies. As mentioned above, the production of ROS upon IL-1β, TNF-α and LPS stimulation has emerged as evidence, but their role in NF-κB activation is still controversial. Recent studies indicate that early ROS production after IL-1β and LPS stimulation is a key messenger for subsequent NF-κB activation, whereas ROS produced after TNFR1 engagement appear slower and would only account for cross-talk between NF-κB and JNK in terms of pro- or anti-apoptotic response. It thus appears that ROS might be important mediators triggering cell life or death, and future studies will have to focus on

identifying what mechanisms are involved in ROS production and accumulation upon TNFα stimulation, which will be important in developing new strategies to prevent excessive cell death under pathological conditions.

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