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Review

Reactive oxygen species mediate crosstalk between NF-κB and JNK

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Abstract

The activation of NF-κB inhibits apoptosis via a mechanism involving upregulation of various antiapoptotic genes, such as *cellular FLICE-inhibitory protein (c-FLIP), Bcl-x_L, A1/Bfl-1,* and *X chromosome-liked inhibitor of apoptosis (XIAP).* In contrast, the activation of c-Jun N-terminal kinase (JNK) promotes apoptosis in a manner that is dependent on the cell type and the context of the stimulus. Recent studies have indicated that one of the antiapoptotic functions of NF-κB is to downregulate JNK activation. Further studies have also revealed that NF-κB inhibits JNK activation by suppressing accumulation of reactive oxygen species (ROS). In this review, we will focus on the signaling crosstalk between the NF-κB and JNK cascades via ROS.

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Keywords: NF-κB; c-Jun N-terminal kinase (JNK); reactive oxygen species (ROS); apoptosis; antiapoptotic genes; necrosis

Abbreviations: JNK, c-Jun N-terminal kinase; c-FLIP, cellular FLICE-inhibitory protein; XIAP, X chromosome-linked inhibitor of apoptosis; ROS, reactive oxygen species; TNF, tumor necrosis factor; IL-1, interleukin-1; IκB, inhibitor of κB; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; ASK1, apoptosis-signal regulating kinase 1; MEKK, MAP/ERK kinase kinase; TAK1, TGFβ-activated kinase 1; MEFs, murine embryonic fibroblasts; TRAF, TNF rceptor-associated factor; c-IAP, cellular inhibitor of apoptosis; ES, embrynonic stem; GADD, growth arrest and DNA damage-inducing protein; SOD, superoxide dismutase; GPx, glutathione peroxidase; PRx, peroxiredoxin; NOX, NADPH oxidase; BHA, butylated hydroxylanisole; NAC, *N*-acetyl cystein; MKP, MAP kinase phosphatase; MnSOD, manganese-dependent SOD; FHC, ferritin heavy chain; mPT, membrane permeability transition; AIF, apoptosis-inducing factor; HSP, heat shock protein; CHX, cycloheximide

Introduction

NF-κB is a collective term used to describe members of the Rel family of dimeric transcription factors.[1,2] The Rel family regulates transcription of a large number of genes that control cell survival and differentiation including various proinflammatory cytokines, chemokines, and adhesion molecules. Many of these same proinflammatory molecules, including cytokines such as tumor necrosis factor (TNF)α and interleukin-1 (IL-1), are able to activate NF-κB, initiating a signaling cascade of activation. NF-κB can also be activated by Toll-like receptors that recognize pathogen-associated molecules or by cellular stress induced following UV or γ-irradiation. The recent identification of molecules, which regulate the activation of the NF-κB heterodimer, RelA(p65) and p50 has enhanced our understanding of the molecular mechanisms controlling inflammation (Figure 1). Signaling systems induced by a variety of stimuli activate two serine kinases, termed IκB kinase (IKK)α and IKKβ (or IKK1; IKK2), which target the inhibitors of κB (IκB). The subsequent phosphorylation by these kinases leads to eventual ubiquitination and proteasome-dependent degradation of IκB, releasing the latent dimeric transcription factor to the nucleus. A key mechanism by which NF-κB controls cell survival[3–5] is to enhance transcription of various antiapoptotic genes, including *cellular FLICE-inhibitory protein (c-FLIP), Bcl-x_L, A1 (also known as Bfl-1), and XIAP (X chromosome-liked inhibitor of apoptosis).*[6,7]

Regulation of cell death and survival is also controlled in part by another signaling cascade activated by the mitogen-activated protein kinase (MAPK), which is induced following cellular stress or cytokine signaling.[8,9] In mammals, the MAPK cascades are composed of three distinct signaling modules, the c-Jun N-terminal kinase (JNK) cascade, the p38MAPK cascade, and the extracellular signal-regulated kinase (ERK) cascade. Each MAPK is activated by sequential protein phosphorylation through a MAPK module; for example MAPK kinase kinase (MAPKKK) phosphorylates MAPK kinase (MAPKK), which in turn phosphorylates MAPK (Figure 2). In the case of the JNK cascade, the MAPKKKs include apoptosis-signal regulating kinase (ASK)1, MAP/ERK kinase kinase (MEKK)s, MTK1 (also known as MEKK4), and TGFβ-activated kinase (TAK)1. These MAPKKKs activate MKK4 and/or MKK7, which then in turn activate JNK, the targets of which include the AP1-related transcription factors, such as c-Jun.[8,9] Cytokines and growth factors including TNFα and IL-1 induce rapid (within 10 min) yet transient activation of MAPK, whereas cellular stresses, such as UV or γ-irradiation, induce prolonged MAPK activation. Several lines of evidence suggest that transient MAPK activation is associated with gene expression, proliferation, and differentiation, whereas prolonged MAPK activation promotes cell

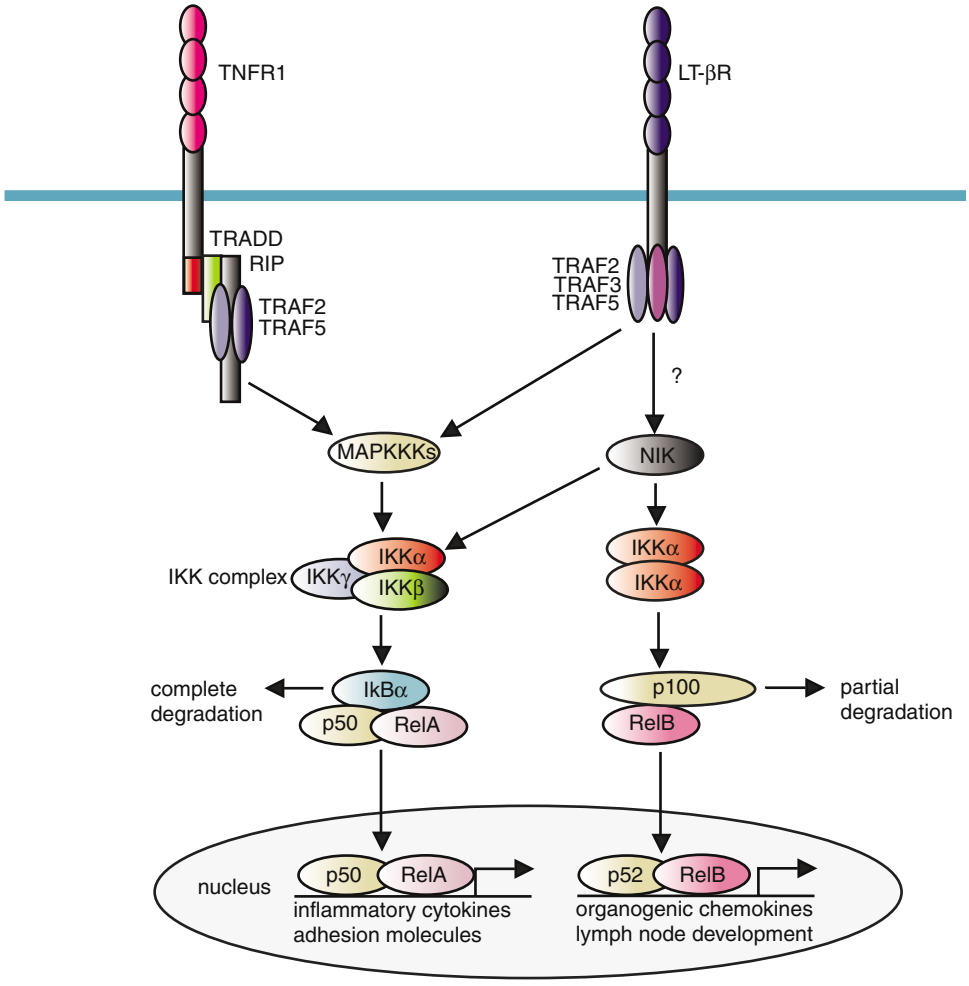

Figure 1 The classical and nonclassical NF-κB activation pathways. The classical NF-κB pathway is activated by inflammatory cytokines including TNFα and IL-1. Activation of the classical pathway depends on TRAFs, MAPKKKs including TAK1 and MEKK3, and the IKK complex containing IKKβ and IKKγ subunits. Activation of the IKK complex results in degradation of the inhibitor protein, IκBα and subsequent nuclear translocation of RelA/p50 dimers. The classical pathway mediates coordinate expression of inflammatory cytokines and adhesion molecules. The nonclassical pathway induces nuclear translocation of RelB/p52 dimers, is strictly dependent on IKKα homodimers and is activated by members of the TNF receptor family, such as lymphotoxin-β receptor (LT-βR) and CD40 via NF-κB-inducing kinase (NIK). NIK is also involved in activation of the classical pathway by CD27 and CD40, but not TNFR. The nonclassical pathway plays a central role in the expression of genes involved in development and maintenance of secondary lymphoid organs. The roles of TRAFs in activation of the nonclassical pathway remain unclear

death, by a mechanism that does not solely involve gene activation, and is cell type- and stimuli-dependent[10,11]

Pro- and Antiapoptotic Roles of JNK

Although the activation mechanisms of JNK have been extensively investigated, the biological consequence of JNK activation in cell death is still controversial.[7,8,12,13] The most convincing evidence that JNK signaling promotes apoptosis comes from the experiments using mice deficient in the JNK activation cascade. In JNK1 and JNK2 double knockout mice, neuronal apoptosis is suppressed in the hindbrain, but increased in the forebrain, indicating that both JNK1 and JNK2 regulate region-specific apoptosis during early brain development.[14] Moreover, murine embryonic fibroblasts (MEFs) from JNK1 and JNK2 double knockout mice are resistant to apoptosis induced by genotoxic stress including exposure to anisomycin, methylmethanesulfonate, and UV,[15] although a recent study has challenged this conclusion.[16] Consistent with these results, primary neurons from both neuron-specific JNK3 isoform knockout mice, and knockin mice expressing the nonphosphorylated form of c-Jun (c-Jun^{AA}), are resistant to excitotoxic glutamate-receptor agonist, kainate-induced apoptosis.[17,18] Moreover, MEFs from ASK1 knockout mice exhibit decreased sensitivity to TNFα- and H_2O_2-induced apoptosis.[19]

Several lines of evidence demonstrate that the proapoptotic JNK cascade ultimately induces apoptosis via the mitochondria-dependent pathway. JNK phosphorylates members of the Bcl-2 family of proteins, such as Bcl-2 and Bcl-x_L, and inactivates their antiapoptotic function.[20–24] Moreover, the ectopic expression of constitutively active JNK (using the MKK7-JNK1 fusion protein) efficiently induces apoptosis in wild-type cells, but not cells lacking the proapoptotic Bcl-2 family members, Bax and Bak, which are essential for the

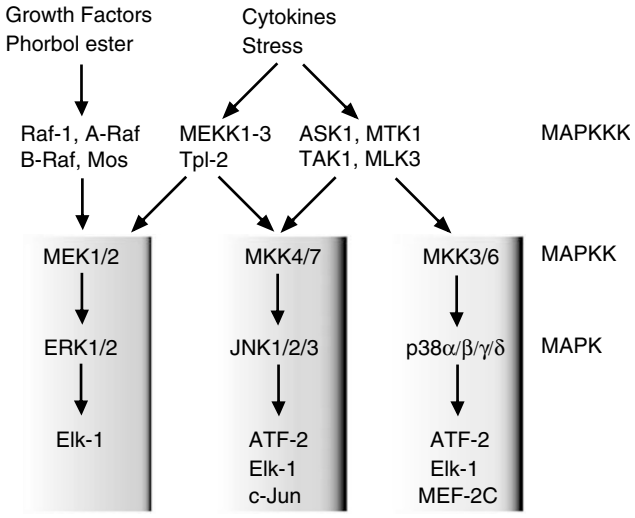

Figure 2 Three MAP kinase modules in mammals. Upon stimulation with growth factors, cytokines, or various inducers of cell stress, MAPKKKs are activated and subsequently phosphorylate MAPKKs, which in turn activate MAPKs. Activated MAPKs subsequently phosphorylate specific transcription factors and activate their transcriptional activity

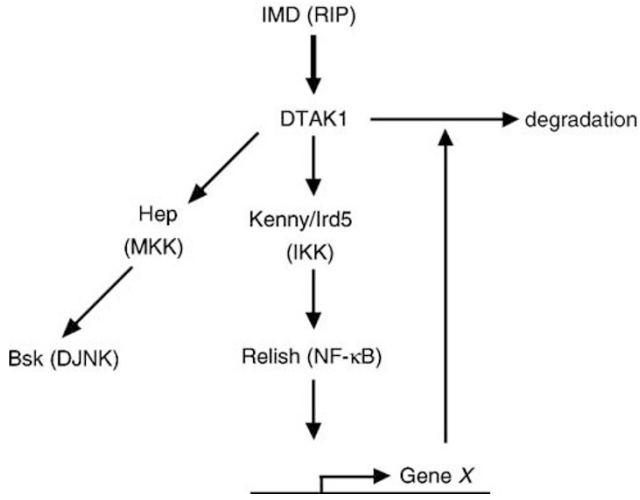

Figure 3 Crosstalk between NF-κB and JNK in the *Drosophila* IMD pathway. IMD (a homologue of RIP) activates DTAK1, which in turn activates DJNK and Relish (a homologue of NF-κB). Unidentified gene(s) (X) induced by Relish mediate the degradation of DTAK1, limiting the duration of JNK activation

mitochondria-dependent apoptotic pathway.[25] Furthermore, JNK activates proapoptotic members of the Bcl-2 family, Bim and Bmf, resulting in activation of Bax and Bak.[26] Recently, Deng *et al*.[27] revealed an unexpected role of JNK in the induction of the caspase 8-independent cleavage of Bid. Under conditions in which TNFα-induced NF-κB activation is blocked, JNK induces caspase 8-independent cleavage of Bid at a different site, resulting in the production of jBid and not the previously described tBid. jBid translocates to the mitochondria leading to the preferential release of Smac (also known as DIABLO). Smac then disrupts a complex consisting of the TNF receptor-associated factor (TRAF)2 and the cellular inhibitors of apoptosis (c-IAPs) complex, resulting in caspase 8 activation and ultimately the induction of apoptosis. Finally, Tsuruta *et al*.[28] have recently reported that JNK phosphorylates the 14-3-3 protein, a cytoplasmic anchor of Bax, and that phosphorylated 14-3-3 fails to sequestrate Bax into the cytoplasm, therefore inhibiting its translocation to the mitochondria.

In contrast to the proapoptotic function of JNK as described above, numerous studies demonstrate an antiapoptotic role for JNK. Nishina *et al*.[29,30] have illustrated that MKK4 knockout mice die due to massive hepatocyte apoptosis, and that MKK4-deficient T cells exhibit increased sensitivity to anti-Fas and anti-CD3-induced apoptosis, indicating that the JNK pathway mediates survival signals. Furthermore, differentiated embryonic stem (ES) cells lacking MEKK1 showed reduced oxidative stress-induced JNK activation and were more susceptible to apoptosis.[31] Moreover, Lamb *et al*.[32] have reported that JNK1 and JNK2 double knockout MEFs show increased sensitivity to TNFα-induced cell death, and that this increased sensitivity is due to defective JNK-mediated upregulation of c-IAP2. Finally, Yu *et al*.[33] reported that JNK phosphorylates the Bcl-2 family protein, BAD and inactivates its proapoptotic function. Collectively, these data suggest that under certain experimental conditions JNK can protect cells from apoptosis.

To explain the apparently controversial findings described above, factor(s) other than JNK activation should be taken into account, such as the activation of other signaling cascades branching from the JNK pathway, including NF-κB. Indeed, many stimuli such as TNF-related cytokines, simultaneously activate both JNK and NF-κB pathways, but do not usually induce apoptosis in normal cells. In contrast, genotoxic stress preferentially activates the JNK pathway with marginal activation of NF-κB, and thus apoptosis predominates. Although genotoxic stress induces translocation of NF-κB, the NF-κB complex containing RelA/p50 heterodimer turns out to be transcriptionally inactive.[34] Thus, it is reasonable to speculate that molecules that are regulated by NF-κB, could critically affect cell fate induced by the JNK cascade.

NF-κB Downregulates JNK

In the past, the contributions of the NF-κB and JNK pathways to cell death have been discussed independently. However, two recent studies have revealed signaling crosstalk between the NF-κB and JNK pathways. Tang *et al*.[35] and De Smaele *et al*.[36] have independently demonstrated that TNFα induces prolonged JNK activation in NF-κB activation-deficient cells, such as RelA and IKKβ knockouts, and cells stably expressing degradation-resistant IκBα. Consistent with previous studies,[10,11] this prolonged JNK activation was found to promotes apoptosis, suggesting that gene(s) are induced by TNFα in an NF-κB-dependent fashion normally block JNK activation. Two target genes that they identified which block JNK activation were growth arrest and DNA damage-inducing protein (GADD45)β and XIAP. Given that GADD45β was known to interact with and activate MTK1/MEKK4, (which triggers the p38 and JNK pathways),[37] its inhibitory effect on

JNK activation was unexpected. The other molecule identified, XIAP, had previously been shown to inhibit apoptosis by inhibiting activation of caspases by direct binding,[38] thus, in this study Tang *et al*.[35] reveal a novel antiapoptotic function of XIAP. Furthermore, Papa *et al*.[39] showed that GADD45β binds to and inhibits the JNK activator, MKK7 through the competitive inhibition of ATP. However, this inhibitory action of GADD45β is cell-type specific, since TNFα-induced JNK activation is not prolonged in GADD45β knockout MEFs or splenocytes.[40] Collectively, these studies demonstrate a cell-type specific molecular link between NF-κB and JNK.

In *Drosophila*, there are several counterparts of signaling components in the mammalian NF-κB and MAPK pathways,[41] such as DJNK, a homologue of JNK; IMD, a homologue of RIP; TAK1, a MAPKKK that activates JNK; and the NF-κB homologue, Relish (Figure 3). The biological consequences of the JNK pathway (DJNK) in *Drosophila* are less complicated than in mammals, functioning to preferentially promote apoptosis. Park *et al*.[42] demonstrated that JNK activation is prolonged in S2 cells lacking Relish. Moreover, they showed that Relish activation leads to degradation of TAK1, resulting in termination of JNK signaling. These results indicate that the regulatory crosstalk between the JNK and NF-κB pathways is also conserved in *Drosophila*. Interestingly, two recent papers have demonstrated that the phosphorylated form of c-Jun is recognized by a specific ubiquitin ligase and is then subsequently degraded by the ubiquitin-proteasome pathway.[43,44] This indicates that apoptotic c-Jun-dependent transcription is negatively regulated by the ubiquitin-proteasome pathway. Although it is currently unknown whether this proteasome-dependent c-Jun degradation pathway is regulated by NF-κB, this system is reminiscent of degradation of TAK1 by Relish in the *Drosophila* IMD pathway.

Reactive Oxygen Species – Emerging Mediators of Prolonged JNK Activation

The two studies described above have convincingly demonstrated that NF-κB downregulates JNK activation and have identified candidate molecules that inhibit JNK activation. Nevertheless, the molecular mechanisms underlying the NF-κB-dependent inhibition have still been controversial. Reactive oxygen species (ROS) have emerged as bridging molecules mediating the crosstalk between NF-κB and JNK.[45–47] ROS, including superoxide anions, hydrogen peroxide and hydroxyl radicals, are accidentally generated in the mitochondria during the transport of electrons from the reducing equivalent (NADH-$FADH_2$) to molecular oxygens through a mitochondrial respiratory chain of enzymatic complexes (I–IV) (Figure 4a).[48,49] Under normal physiological conditions, ROS are rapidly eliminated by antioxidant enzymes, including superoxide dismutases (SODs), catalase, glutathione peroxidase (GPx), and peroxiredoxin (PRx) (Figure 4b).[48,49] Dysregulation of electron transport through the mitochondrial respiratory chain or an impairment in the function of antioxidant enzymes results in the accumulation of

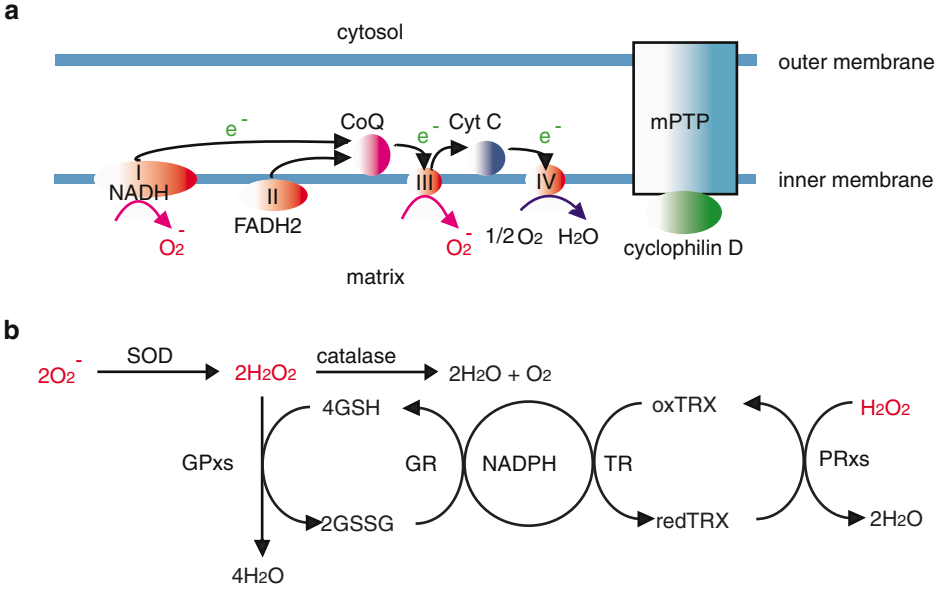

Figure 4 Generation of ROS in the mitochondria and their elimination by cellular antioxidants. (**a**) The mitochondrial respiratory chain consists of four multimeric complexes (complexes I–IV), coenzyme Q (CoQ), and cytochorome *c* (Cyt *C*). Electrons (e^-) are transferred from the reducing equivalent (NADH-$FADH_2$) to molecular oxygens through the mitochondrial respiratory chain, finally generating water at complex IV. During the electron transfer, reactive oxygen species (ROS) are accidentally generated at complexes I and III. The mitochondrial permeability transition pore (mPTP) is regulated by cyclophilin D, and opened by high calcium or oxidative stress. Opening of this pore results in massive loss of ions and metabolites from the matrix (see the text). (**b**) O_2^- is converted into H_2O_2 by superoxide dismutases (SODs). H_2O_2 is then eliminated by catalase, glutathione peroxidases (GPxs), and peroxiredoxins (PRxs). During elimination of H_2O_2, reduced glutathione (GSH) is converted to disulfide form (GSSG) by GPxs, and then GSSG is recycled to GSH by glutathione reductase (GR). However, PRxs also catalyze H_2O_2 into H_2O by using reduced thioredoxin (TRx). Oxydized TRx is then recycled back to redTRx by thioredoxin reductase (TR). NADPH is essential for both recycling reactions. ROS are indicated using red characters

ROS. In addition to the byproducts of ROS which accumulate in the mitochondria as described above, NADPH oxidase (NOX) enzymes, which are localized in the plasma membrane have also been identified as producers of ROS in various types of cells in response to growth factors, cytokines, or calcium signals.[50] Moreover, several enzymes localized in the peroxisomes, cytoplasm, and endoplasmic reticulum may also generate ROS.[48,49,51] Whether ROS play a central role in cytokine-induced MAPK activation under physiological conditions, however, remains controversial.

Previous studies have shown that ROS directly activate various kinases, including ASK1, MEKK1, c-src, EGFR, and PDGFR, which in turn activate the MAPK cascades.[52] Consistent with a previous study,[19] we and others[45,53–55] have demonstrated that NF-κB downregulates JNK activation by suppressing TNFα-induced ROS accumulation. Notably, TNFα induces early and transient JNK activation in wild-type cells, whereas TNFα induces ROS accumulation leading to prolonged JNK activation in NF-κB activation-deficient cells. In accordance with these findings, prolonged JNK activation is inhibited by pretreatment of cells with antioxidants such as butylated hydroxylanisole (BHA) and *N*-acetyl cysteine (NAC), suggesting that the mechanisms of early/transient and prolonged JNK activation are qualitatively different. Moreover, extended JNK activation is still induced in TRAF2/TRAF5 double KO cells,[45] in which TNFα-induced early/transient JNK activation is severely impaired.[56] Collectively, these results demonstrate that early/transient JNK activation is dependent upon TRAF, whereas prolonged JNK activation is ROS-dependent.

Another important issue is how ROS induce long-lasting JNK activation. A previous study[57] has shown that ASK1 responds to ROS and triggers the JNK and p38MAPK, but not ERK cascades. Given that TNFα and arsenic induce both prolonged JNK and ERK activation in RelA KO and IKKβ KO MEFs,[45,58] a kinase that activates the ERK cascade, such as MEKK1 may also be involved in this prolonged MAPK activation. In addition, as ROS directly activate JNK, we need to consider the possibility that ROS may inactivate inhibitors, which normally suppress JNK activation, therefore resulting in prolonged JNK activation. In this respect, Kamata *et al*.[55] reported that ROS inactivate MAP kinase phosphatases (MKPs)[59] by oxidizing cysteine residues critical for their phosphatase activities. Moreover, oxidized MKPs are rapidly degraded by the ubiquitin-proteasome pathway. Collectively, these data suggest that ROS may utilize two different mechanisms in order to promote persistent JNK activation. ROS may either positively activate MAPKKKs, resulting in JNK activation, and/or inactivate MKPs that would otherwise dephosphorylate and inactivate JNK.

In addition to ROS, we do not formally exclude the possibility that activation of the caspase cascades also contributes to JNK activation. In this respect, several kinases, including MEKK1,[60] MST,[61] and PAK2,[62] have been reported to be cleaved by caspases, resulting in their activation. Under the conditions in which NF-κB activation is impaired, TNFα stimulation induces both caspase-dependent apoptosis and ROS-dependent necrosis. Therefore, activation of the caspase cascades may contribute to JNK activation in a stimuli-dependent fashion.

The Molecular Mechanisms of TNFα-Induced ROS Accumulation

Regarding the mechanism whereby TNFα induces ROS accumulation in NF-κB activation-deficient, but not wild-type cells, two possibilities need to be considered. Firstly, impaired induction of antioxidant enzymes or antioxidants that are induced by NF-κB under normal conditions might be responsible for ROS accumulation. Indeed, previous studies[63,64] have shown that various antioxidant enzyme genes including *manganese-dependent SOD (MnSOD)*, *metallothionein*, *glutathione S-transferase*, and *ferritin heavy chain (fhc),* are induced by TNFα in an NF-κB-dependent fashion. Pham *et al*.[54] and Kamata *et al*.[55] have also shown that the ectopic expression of *fhc* and *MnSOD* inhibits TNFα-induced ROS accumulation in RelA KO and IKKβ KO cells, respectively. However, given that complicated and multiple step reactions might be required for efficient elimination of ROS (Figure 5b), it is rather surprising that expression of a single gene, such as *fhc* or *MnSOD* is sufficient for ROS elimination. In fact, the inhibitory effect of MnSOD on TNFα-induced ROS accumulation is not complete,[55] indicating that another molecule or mechanism might also be involved in this process.

Another possibility is that latent signaling cascade(s) that are normally suppressed by NF-κB, may dominate over its inhibition and therefore induce ROS. Chen *et al*.[58] have reported that TNFα promotes expression of a member of the p450 family, *cyp1b1* that generates ROS in IKKβ KO cells. Moreover, several studies have shown that ROS accumulation is induced during apoptotic processes. A recent study[65] indicated that activated caspase 3 cleaves the p75 subunit of complex I of the mitochondrial electron transport chain, resulting in ROS accumulation. Similarly, Giorgio *et al*.[66] have shown that proapoptotic signals induce release of $p66^{Shc}$ from a putative inhibitory complex, which in turn oxidizes reduced cytochrome c, thereby generating ROS. On the other hand, Ventura *et al*.[53] have reported that TNFα-induced ROS accumulation is abolished in cells lacking JNK1 and JNK2, indicating that a central role for JNK in ROS accumulation. Given that ROS promote JNK activation, they hypothesized that activation of the JNK pathway induces ROS accumulation, which can in turn activate JNK in a positive feedback fashion. However, it remains unclear how JNK induces ROS accumulation. It is reasonable to surmise that the molecular mechanisms underlying TNFα-induced ROS accumulation are not due to a single mechanism, but are more likely to be cell-type specific. To determine the subcellular localization of ROS generation, such as the mitochondria, cytoplasm, or plasma membrane might provide valuable information needed to elucidate the mechanisms by which TNFα induces ROS. Given that there is currently no reliable way to determine the subcellular location of ROS generation using oxidation-sensitive dyes, it is crucial to develop such detection systems to better understand the signaling specificity induced by various oxidative stresses as well as the mechanism whereby TNFα induces ROS.

Do ROS Induce Apoptosis or Necrosis?

Under various pathological conditions such as ischemia, excessive amounts of accumulated ROS induce apoptosis or

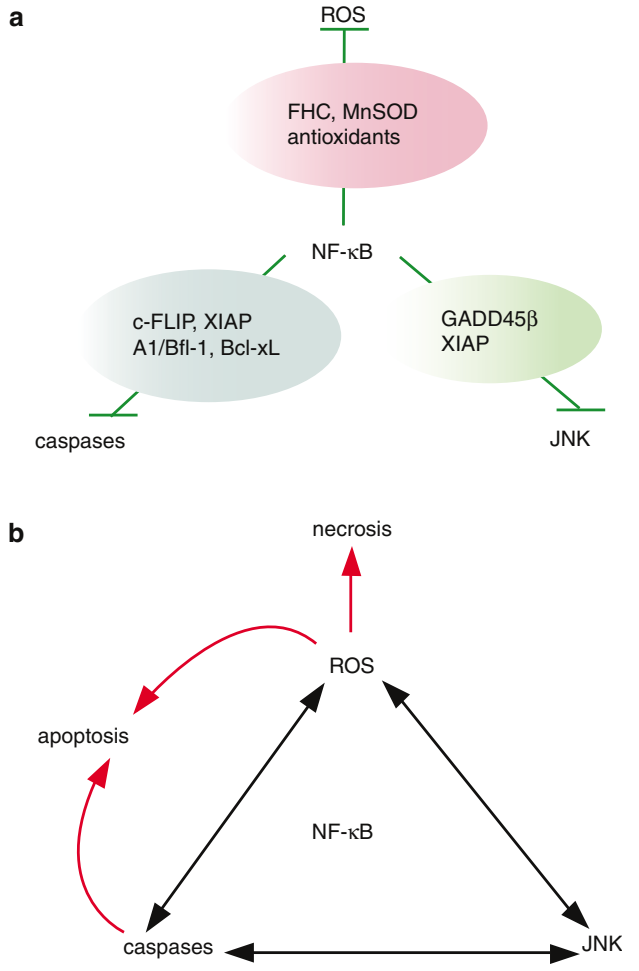

Figure 5 Signaling crosstalk between NF-κB and JNK. (**a**) Activation of NF-κB by TNFα induces expression of GADD45β and XIAP that downregulates JNK activation. NF-κB also induces expression of c-FLIP, Bcl-x_L, and A1/Bfl-1 that inhibit caspase activation. Furthermore, NF-κB inhibits ROS accumulation by upregulating FHC and MnSOD. Under these conditions, TNFα induces transient JNK activation and cell survival. (**b**) In cells unresponsive to NF-κB activation induced by TNFα (TRAF2/TRAF5, RelA, and IKKβ KO cells), JNK activation is prolonged and caspase activation is induced due to the lack of induction of these molecules. Furthermore, ROS also promote JNK activation. Through the coordinate activation of these pathways, TNFα induces apoptosis and necrosis

necrosis by activating the MAPK, caspase cascades, and /or by disrupting mitochondrial membrane potential.[67] This contribution of ROS to apoptosis and necrosis is highly cell-type specific, and also depends on the amount of endogenously or exogenously generated ROS present. As ROS induce activation of the JNK cascade, apoptosis induced by ROS is likely to be dependent on the JNK-mediated mitochondria-dependent apoptotic pathway. Alternatively, ROS may act directly on the mitochondria, inducing the mitochondrial membrane permeability transition (mPT) and resulting in the release of apoptogenic factors, such as cytochrome *c*, apoptosis-inducing factor (AIF), and/or Smac/DIABLO.[68,69]

The mechanisms underlying ROS-induced necrosis have been highly debated. It is well known that TNFα induces ROS-dependent necrosis in murine fibrosarcoma, L929 cells.[70] Geldanamycin treatment induces the degradation of heat shock protein (HSP)90 and its client protein, RIP, causing a shift from necrosis to apoptosis in L929 cells, indicating an essential role for RIP in TNFα-induced necrosis.[71] Similarly, Holler *et al*.[72] have also reported that a critical role for RIP in Fas-, TNFα-, and TRAIL-induced necrotic cell death. The contribution of ROS to receptor-mediated necrosis was not investigated in these studies; however, and the conclusion reached was that FADD and kinase activity of RIP, but not caspase 8, are essential for necrosis. Intriguingly, a recent report[73] has highlighted that both cell death followed by ischemic brain injury and RIP-dependent necrotic cell death are tightly linked with autophagy. However, further studies will be required to address the role of autophagy in necrotic cell death.

Two recent studies[74,75] have shown that H_2O_2-induced necrosis, but not genotoxic stress-induced apoptosis is reduced in cells lacking cyclophilin D. As mPT induced by H_2O_2 is also severely impaired in cyclophilin D knockout cells, it appears that mPT is crucial for H_2O_2-induced necrosis. Therefore, one of the mechanisms of ROS-induced necrosis may be the opening of an mPT pore, resulting in the loss of membrane potential and causing extensive swelling of the mitochondria. However, it is currently unknown whether all RIP- and ROS-dependent pathways leading to necrotic cell death finally converge on mPT. To investigate this matter further, it would be interesting to test whether knockdown of cyclophilin D suppresses TNFα-induced ROS-dependent necrosis in L929 cells or NF-κB activation-deficient cells.

Finally, we need to further our understanding of factor(s) that may affect the fate of cells exposed to ROS. Protein synthesis inhibitors, such as cycloheximide (CHX) and emetine, which are usually required for TNFα to induce cell death in wild-type cells, might affect the fate of the TNFα-stimulated cells and determine whether they die from apoptosis or necrosis. Indeed, TNFα-induced necrotic cell death is preferentially observed in cells that are stimulated with TNFα alone,[53] and not in those stimulated with TNFα plus CHX or emetine.[53,54] Therefore, TNFα-induced apoptosis might prevail over necrosis in the presence of protein synthesis inhibitors,[76] although the detailed molecular mechanism remains unknown.

Concluding Remarks

Recent advances in gene targeting techniques convincingly demonstrate the proapoptotic and antiapoptotic function of the JNK signaling cascade. Although the caspase cascade is sufficient for the induction of apoptosis, the activation of the JNK pathway itself does not appear to be sufficient for determining cell fate. As Lin[12] describes, the JNK cascade appears to regulate the path to cell death or survival. In this respect, the central checkpoint at which cell fate is determined involves NF-κB. As described in Figure 5a, activation of NF-κB is sufficient for inhibiting the cascades induced by proapoptosis-inducing molecules, caspases, JNK, and ROS in normal cells. Under the conditions, in which NF-κB-mediated survival signals are blocked (such as cellular parasitism by viruses and other pathogens, or genotoxic

stress), JNK and ROS promote cell death in a context-dependent manner (Figure 5b). More importantly, several studies indicate that treatment of cells with caspase inhibitors enhances ROS-dependent necrosis both *in vitro* and *in vivo*.[70,77] To understand the NF-κB-mediated survival signals in more detail and to develop novel strategies to prevent excessive cell death under the pathological conditions, future studies will focus on identifying the molecules involved in JNK activation and ROS accumulation.

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