ROS mediate signaling crosstalk between NF-κB and JNK

Review Article

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Summary

Activation of NF-κB inhibits apoptosis by upregulating various anti-apoptotic genes, such as c-FLIP, Bcl-x$_L$, A1/Bfl-1, and X chromosome-liked inhibitor apoptosis (XIAP). However, the molecular mechanism by which NF-κB inhibits apoptosis is not fully understood. In contrast, activation of c-Jun N-terminal kinase (JNK) promotes apoptosis in a context-dependent manner. Recent studies showed that one of the anti-apoptotic functions of NF-κB is to downregulate JNK activation, and this function is mediated by two NF-κB-inducible genes, GADD45β and XIAP. More recently, NF-κB activation inhibits TNFα-induced accumulation of reactive oxygen species (ROS) that mediates prolonged JNK activation and necrotic cell death. In this review, we will focus on the signaling crosstalk between NF-κB and JNK cascades via ROS.

I. Introduction

NF-κB is a collective term referring to dimeric transcriptional factors that belong to the Rel family protein, and regulates expression of various inflammatory cytokines, chemokines, and adhesion molecules (Ghosh and Karin, 2002; Ghosh et al, 1998). NF-κB is activated by inflammatory cytokines and cellular stresses, including tumor necrosis factor (TNF) , interleukin-1 (IL-1), lipopolysaccharide (LPS), UV, or γ-irradiation. Recent identification of the components of the IκB kinase complex resulted in a great progress in understanding the molecular mechanism of NF-κB activation (**Figure 1**). In addition to the function of NF-κB to regulate various inflammatory responses, NF-κB, especially RelA (a major component of NF-κB) containing complex, also plays a

pivotal role in protection from TNFα- and genotoxic stress-induced apoptosis (Beg and Baltimore, 1996; Van Antwerp et al, 1996; Wang et al, 1996). Currently, NF-κB-mediated anti-apoptotic functions are considered to upregulate various anti-apoptotic genes, including c-FLIP, Bcl-x$_L$, A1 (also known as Bfl-1), and X chromosome-liked inhibitor apoptosis (XIAP) (Barkett and Gilmore, 1999; Karin and Lin, 2002). However, NF-κB-mediated survival signals are not completely understood.

The mitogen-activated protein kinase (MAPK) cascades are activated by various cellular stresses and growth factors, and are involved in cytokine production, differentiation, proliferation, and cell death (Davis, 2000; Kyriakis and Avruch, 2001). In mammals, MAPK cascades are composed of three distinct signaling modules,

c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK) cascades. Each MAPK is activated by sequential protein phosphorylation through a MAPK kinase module, i.e., MAPKKK MAPKK MAPK (**Figure 2**).
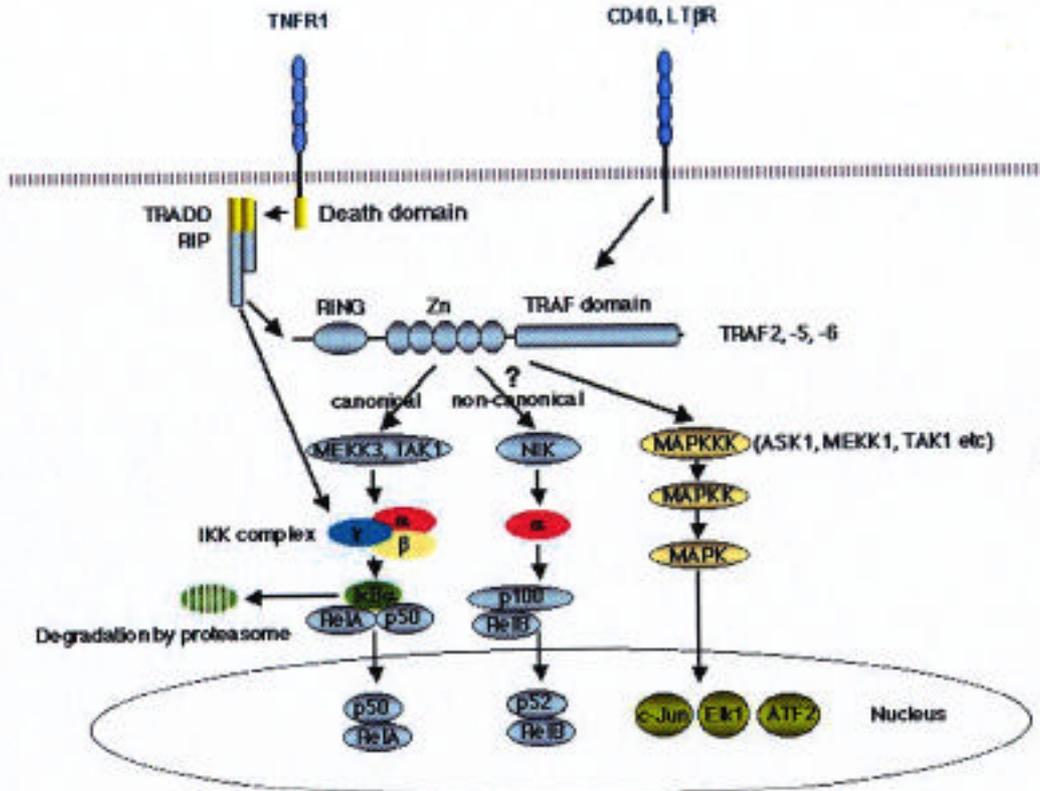

Figure 1. Activation pathways through the TNF receptor superfamily. Oligomerization of the receptors induces recruitment of adaptor molecules, TRAFs, which in turn activate various MAPKKKs. Then, activated MAPKKKs subsequently activate both NF- B and MAPK pathways. Regarding the NF- B pathways, there are two pathways identified, one is canonical and the other is non-canonical. Canonical pathway is dependent on the IKK / / complex, but non-canonical pathway is dependent on IKK , but not or . Target genes induced by these two pathways appear to be different. Contribution of TRAFs to non- canonical pathway is still controversial.
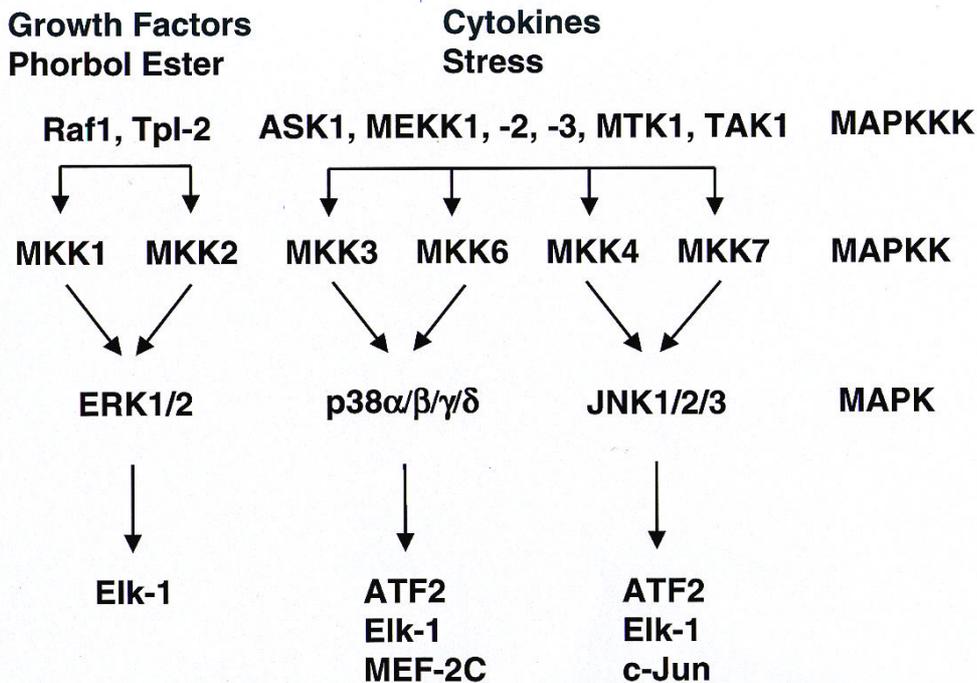

Figure 2. Three MAP kinase modules in mammals. Upon stimulation with growth factors, cytokines, or various stresses, MAPKKKs are activated and subsequently phosphorylate MAPKKs, which in turn activate MAPKs. Activated MAPKs finally phosphorylate specific transcription factors and activate their transcriptional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

Regarding JNK cascade, MAPKKKs including apoptosis-signal regulating kinase (ASK)1, MAP/ERK kinase kinase (MEKK)s, MTK1 (also known as MEKK4), and TGF -activated kinase (TAK)1 activate MKK4 and/or MKK7, which in turn activate JNKs. Finally, activated JNKs phosphorylate specific targets, such as c-Jun and activate their transcriptional activities (Davis, 2000; Kyriakis and Avruch, 2001). Cytokines and growth factors, including TNF and IL-1 induce rapid and transient MAPK activation with a peak at 10 min and then declining to basal level by 60 min. On the other hand, genotoxic stresses, such as UV or -irradiation, induce long-lasting or prolonged MAPK activation. Several lines of evidence suggest that transient MAPK activation is associated with gene expression, proliferation or differentiation, whereas prolonged MAPK activation promotes cell death in a cell type- and stimuli-dependent manner (Chen et al, 1996; Guo et al, 1998).

II. Pro- and anti-apoptotic roles of JNK

Although activation mechanism of JNK have been extensively investigated, biological consequence of JNK activation in cell death is still controversial (Davis, 2000; Karin and Lin, 2002; Lin, 2003; Varfolomeev and Ashkenazi, 2004). The most convincing evidence of pro-apoptotic signaling JNK 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, whereas apoptosis is increased in forebrain, indicating that both JNK1 and JNK2 regulate region-specific apoptosis during early brain development (Kuan et al, 1999). Moreover, murine embryonic fibroblasts (MEFs) from JNK1 and JNK2 double knockout mice are resistant to apoptosis induced by genotoxic stresses, including anisomycin, methylmethanesulfonate, and UV (Tournier et al, 2000), although a recent study challenged this conclusion (Wada et al, 2004). Consistent with these results, primary neurons from neuron-specific JNK isoform, JNK3 knockout mice and knockin mice with non-phosphorylated form of c-Jun (c-Jun^{AA}) are resistant to excitotoxic glutamate-receptor agonist, kainate-induced apoptosis (Behrens et al, 1999; Yang et al, 1997). Moreover, MEFs from ASK1 knockout mice decreased the sensitivity to TNF , and H $_2$O$_2$-induced apoptosis (Tobiume et al, 2001).

Several lines of evidence demonstrate that pro-apoptotic JNK cascade finally converges on mitochondria-dependent apoptotic pathway. JNK phosphorylates members of the Bcl-2 family of proteins, such as Bcl-2 and Bcl-x$_L$, and inactivate their anti-apoptotic function (Maundrell et al, 1997; Pandey et al, 1999; Yamamoto et al, 1999; Deng et al, 2001; Fan et al, 2000). Moreover, ectopic expression of constitutively active JNK (MKK7-JNK1 fusion protein) efficiently induced apoptosis in wild-type cells, but not cells lacking pro-apoptotic Bcl-2 family members, Bax and Bak, which are essential for mitochondrial-dependent apoptotic pathway (Lei et al, 2002). Furthermore, JNK activates pro-apoptotic members of the Bcl-2 family, Bim and Bmf, resulting in activation

of Bax and Bak (Lei and Davis, 2003). Recently, Deng et al. revealed an unexpected role of JNK to induce caspase 8-independent cleavage of Bid, jBid (Deng et al, 2003). Under the conditions, in which TNF -induced NF- B activation is blocked, JNK induces caspase 8-independent cleavage of Bid at a distinct site from previously described Bid (tBid) to generate jBid. jBid translocates to mitochondria and leads to preferential release of Smac (also known as DIABLO). Then, released Smac/DIABLO disrupts the TRAF2 and c-IAPs complex, resulting in caspase 8 activation and induction of apoptosis. Very recently, Tsuruta et al. reported that JNK phosphorylates 14-3-3 protein, a cytoplasmic anchor of Bax, and phosphorylated 14-3-3 fails to sequester Bax in the cytoplasm, resulting in translocation of Bax to mitochondria (Tsuruta et al, 2004)

As described above for pro-apoptotic function of JNK, numerous studies also demonstrate the anti-apoptotic role of JNK. Nishina et al. showed that MKK4 knockout mice die due to liver apoptosis, and reconstituted T cells show increased sensitivity to anti-Fas and anti-CD3-induced apoptosis, indicating that JNK pathway mediates survival signals (Nishina et al, 1997; Nishina et al, 1999). Differentiated embryonic stem (ES) cells lacking MEKK1 showed a defect of oxidative stress-induced JNK activation and also increased sensitivity to oxidative stress-induced apoptosis (Minamino et al, 1999). Moreover, by using JNK1 and JNK2 double knockout MEFs, Lamb et al. showed that these cells gain sensitivity to TNF -induced cell death and this increased sensitivity is due to the defect of JNK-mediated upregulation of c-IAP2 (Lamb et al, 2003). Furthermore, Yu et al. reported that JNK phosphorylates the Bcl-2 family protein, BAD and inactivates its pro-apoptotic function (Yu et al, 2004).

To integrate these apparently controversial findings described above together, factor(s) other than JNK activation should be considered, including activation of other signaling cascade branching from the JNK pathway, such as NF- B. Indeed, many stimuli, such as TNF related cytokines, simultaneously activate both JNK and NF- B cascades, but do not usually induce apoptosis in normal cells. In contrast, genotoxic stresses preferentially activate JNK cascade with marginal activation of NF- B, and thus apoptosis predominates. Although genotoxic stresses induce translocation of NF- B, the NF- B complex containing RelA/p50 heterodimer turns out to be transcriptionally inactive (Campbell et al, 2004). Thus, it is reasonable to speculate that molecules that are regulated by NF- B, could critically affect cell fate induced by JNK cascade.

III. NF- B downregulates JNK activation

Until recently, contribution of NF- B and JNK cascades to cell death has been discussed independently, but two recent papers have revealed signaling crosstalk between NF- B and JNK cascades. Tang et al. and De Smaele et al. independently demonstrated that TNF induces prolonged JNK activation in NF- B activation-deficient cells [RelA knockout, IKK (an essential

component of the IKK complex) knockout, or cells stably expressing degradation-resistant IκB] and this prolonged JNK activation promotes apoptosis (De Smaele et al, 2001; Tang et al, 2001) (**Figure 3A and C**). These results indicate that genes are induced by TNF in NF-κB-dependent fashion, normally block activation of JNK. By searching such genes, they identified GADD45β and XIAP as candidates to block JNK activation. Since GADD45β was identified in an earlier study as interacting and activation molecule of MTK1/MEKK4 that triggers p38 and JNK pathways (Takekawa and Saito, 1998), inhibitory effect of GADD45β on JNK activation appears to be unexpected. On the other hand, XIAP has been show to inhibit apoptosis by inhibiting activation of caspases by direct binding (Deveraux et al, 1997), thus, Tang et al. reveal a novel anti-apoptotic function of XIAP. Collectively, these studies demonstrate a molecular link between NF-κB and JNK, although the detailed molecular mechanism by which GADD45β and XIAP inhibit JNK activation is not clarified in these studies.

In this regard, Papa et al. have elucidated the molecular mechanism how GADD45β inhibits JNK activation (Papa et al, 2004). They identified MKK7 as an interacting molecule of GADD45β. A previous study showed that MKK7 play major role in TNFα-induced JNK activation (Tournier et al, 2001). GADD45β binds to and inhibits MKK7's kinase activity as competitive inhibition of ATP. They also generated cell permeable (TAT linked) peptides to inhibit the interaction of MKK7 with GADD45β, which rendered wild-type cells sensitive to TNFα-induced apoptosis, indicating that GADD45β normally suppresses TNFα-induced apoptosis by blocking of MKK7 activation. Notably, inhibitory action of GADD45β is rather cell-type specific, since TNFα-induced JNK activation is not prolonged in GADD45β knockout MEFs or splenocytes (Amanullah et al, 2003). These results raise the possibility that molecules other than GADD45β regulate JNK in other types of cells.

In *Drosophila*, there are several counterparts of signaling components in the mammalian NF-κB and MAPK pathways, such as DJNK, a homologue of JNK;

IMD, a homologue of RIP; TAK1, a MAPKKK that activates JNK; and the NF-κB homologue, Relish (Silverman and Maniatis, 2001) (**Figure 4**). Biological consequences of the JNK pathway in Drosophila are less complicated than in mammals, functioning to preferentially promote apoptosis. Park et al. demonstrated that JNK activation is prolonged in S2 cells lacking Relish (Park et al, 2004). 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, recent two papers demonstrate that phosphorylated form of c-Jun is recognized by specific ubiquitin ligase and subsequently degraded by the ubiquitin-proteasome pathway (Nateri et al, 2004; Wertz et al, 2004). This indicates the apoptotic c-Jun-dependent transcription is negatively regulated by the ubiquitin-proteasomal system. 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 *Drosophila* IMD pathway.

IV. Contribution of reactive oxygen species (ROS) to prolonged JNK activation

ROS, including superoxide anions, hydrogen peroxide and hydroxyl radicals, are normally generated in the mitochondria and acts as signaling intermediates (Adler et al, 1999; Thannickal and Fanburg, 2000). Under physiological conditions, generated ROS are rapidly eliminated by antioxidant enzymes, including superoxide dismutases (SODs), catalase, glutathione peroxidases (GPxs), and peroxiredoxins (PRxs) (Thannickal and Fanburg, 2000) (**Figure 5**). However, it remains controversial whether ROS play a critical role in cytokine-induced MAPK activation under physiological conditions.
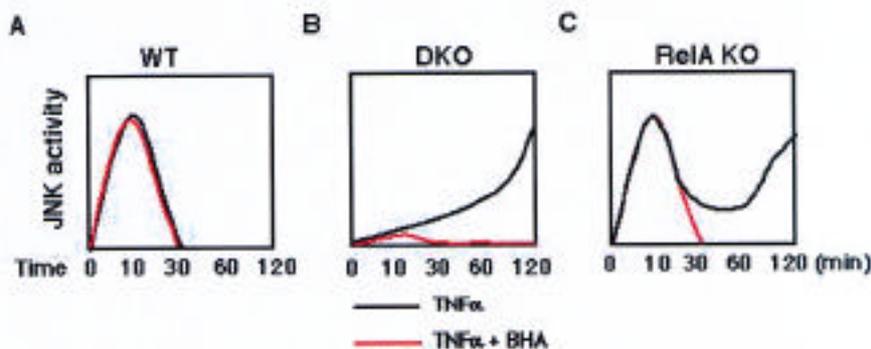

Figure 3. Three different kinetics of JNK activation. Activation patterns of JNK in wild-type (WT) (**A**), TRAF2 and TRAF5 KO (DKO) (**B**), and RelA KO MEFs (**C**). TNF usually induce early/transient JNK activation irrespective in the absence (black line) or presence of antioxidant (red line) in wild-type MEFs. In DKO MEFs, despite the lack of early/transient JNK activation, late/long-lasting JNK activation is induced. In RelA KO MEFs, biphasic JNK activation is induced, early JNK activation is TRAF-dependent and late JNK activation is ROS-dependent.
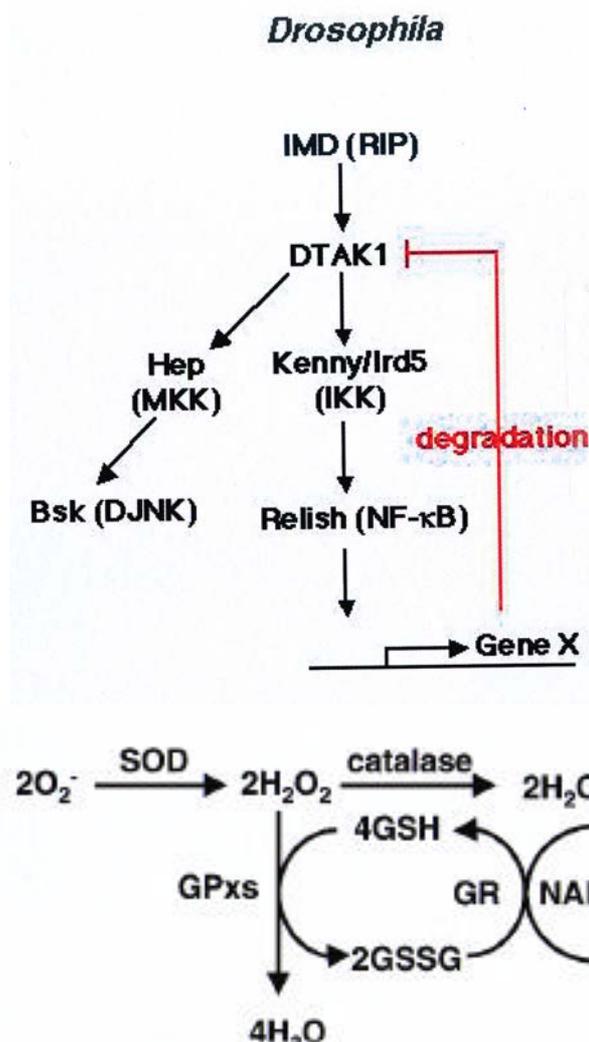
Figure 4. Cross talk of NF- B and JNK in *Drosophila* IMD pathway. IMD (a homologue of RIP) activates DTAK1, which in turn finally activates DJNK and Relish (a homologue of NF- B). Unidentified gene(s) (X) induced by Relish mediate the degradation of DTAK1 and then limit the duration of JNK activation.

Figure 5. Pathways of ROS elimination by cellular antioxidants. O_2^- is converted into H_2O_2 by superoxide dismutases (SODs). Then, H_2O_2 is 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). On the other hand, PRxs also catalyze H_2O_2 into H_2O by using reduced thioredoxin (TRx). Oxydized TRx is recylcled back to redTRx by thioredoxin reductase (TR). NADPH is essential for both recycling reactions.

Previous studies showed that ROS directly or indirectly activate various kinases, including ASK1, MEKK1, c-src, EGFR, and PDGFR, which in turn activate MAPK cascades (Droge, 2002). Recent studies demonstrated that ROS play an important role in prolonged JNK activation in NF- B activation deficient cells. Sakon et al. demonstrated that despite the lack of early JNK activation, late/long-lasting JNK activation is still induced in TNF receptor-associated factor (TRAF)2 and 5 double knockout MEFs (Sakon et al, 2003; Tada et al, 2001) (**Figure 3B**). Interestingly, late/long-lasting JNK activation is completely inhibited in the presence of anti-oxidant, butylated hydroxyanisole (BHA) in DKO and RelA KO MEFs. More importantly, BHA treatment did not inhibit early/transient JNK activation in wild-type or RelA KO MEFs. Consistent with these results, TNF induced ROS accumulation in DKO and RelA KO, but not wild-type MEFs. Together, these results demonstrate that early/transient JNK activation is TRAF-dependent and late/long-lasting JNK activation is ROS-dependent. Chen et al. reported that arsenic treatment induces accumulation

of ROS mediating prolonged JNK activation in IKK knockout MEFs, and this prolonged JNK activation is inhibited by the anti-oxidant (Chen et al, 2003). Similarly, Maeda et al. showed that enhanced ROS accumulation is responsible for TNF -induced prolonged JNK activation in IKK knockout hepatocytes (Maeda et al, 2003). These results suggest mechanism for signaling crosstalk between NF- B and JNK (**Figure 6**).

Although these studies convincingly demonstrate the critical contribution of ROS to prolonged JNK activation in NF- B activation deficient cells, several important questions remain to be solved. How does TNF induce accumulation of ROS in NF- B activation deficient cells? One possibility is that NF- B-inducible genes, such as anti-oxidant enzymes, are rapidly induced by TNF and eliminate ROS. Consistent with this possibility, Sasazuki et al. showed that anti-oxidant enzymes, including superoxide dismutase (SOD)s, glutathione S-transferase, and methallothionein are induced by TNF in wild-type cells, but not DKO cells (Sasazuki et al, 2004). Another possibility is that under conditions in which NF- B
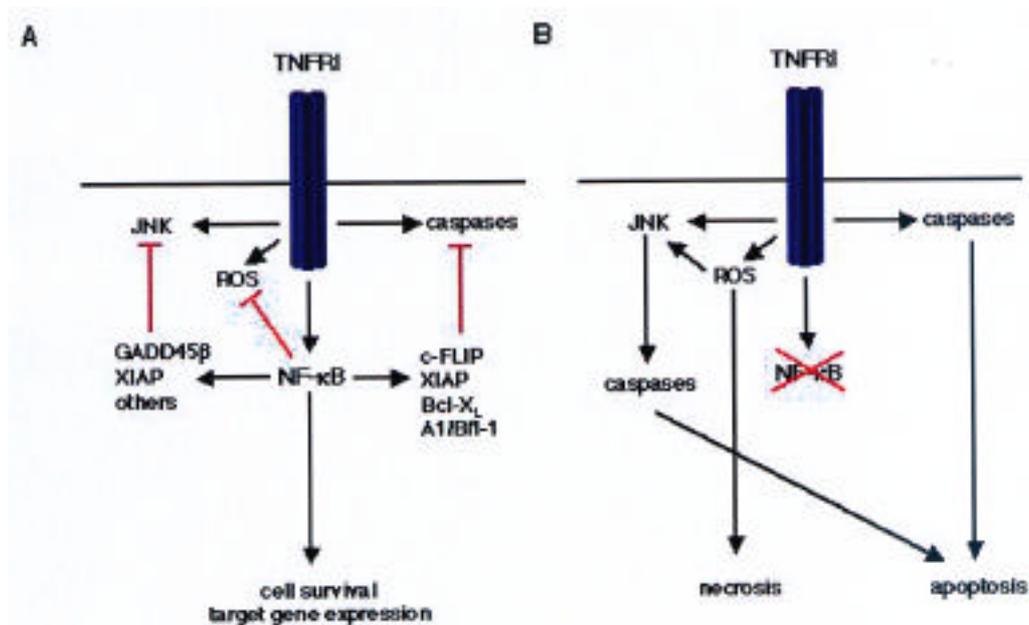
Figure 6. Signaling crosstalk between NF-κB and JNK. (**A**) Activation of NF-κB by TNFα induces expression of GADD45β molecules that downregulate JNK activation. NF-κB also induces expression of c-FLIP, Bcl-x$_L$, and A1/Bfl-1 that inhibit caspase activation. NF-κB inhibits ROS accumulation in undetermined mechanism. Under this condition, TNFα induces transient JNK activation and cell survival. (**B**) In cells lacking NF-κB activation by TNFα (TRAF2 and TRAF5, RelA, and IKKβ KO cells), JNK activation is prolonged and caspase activation is induced due to the lack of induction of GADD45β, XIAP, c-FLIP, Bcl-x$_L$ and A1/Bfl-1. Furthermore, ROS induced by TNFα also contribute to prolonged JNK activation. Through the coordinate activation of these pathways, TNFα induces apoptosis and necrosis.

activation is blocked, TNFα induces upregulation of particular genes that mediate ROS accumulation. Along this line, Chen et al. reported that TNFα induces expression of a member of the p450 family, cyp1b1 that generates ROS (Chen et al, 2003). Another question is the nature of the kinase(s) responsible for the prolonged JNK activation. A previous study showed that ASK1 responds to ROS and triggers JNK and p38MAPK cascades, but not ERK (Ichijo et al, 1997; Saitoh et al, 1998). Given the finding that TNFα and arsenic also induce prolonged ERK activation in RelA KO and IKKβ KO MEFs (Chen et al, 2003; Sakon et al, 2003), a kinase other than ASK1, such as MEKK1 may also be involved in this prolonged MAPK activation.

Most important question is whether ROS-, GADD45β-, and XIAP-mediated regulation of JNK pathway are interconnected. Perhaps the simplest scenario envisions that GADD45β and/or XIAP as inhibitors of ROS accumulation mediated by TNFα. However, this scenario seems to be unlikely, since ectopic expression of GADD45β or XIAP did not inhibit TNFα-induced ROS accumulation in RelA KO MEFs (Sakon et al, 2003). However, the contribution of GADD45β appears to be cell-type specific (Papa et al, 2004), thus GADD45β and ROS may independently inhibit or enhance JNK activation depending on the cell type.

V. Does ROS contribute to TNFα-induced NF-κB activation?

The most controversial and puzzling issue is whether ROS activate NF-κB. In order to evaluate the contribution of ROS to NF-κB activation, it must be appreciated that

the exogenously and endogenously generated ROS may yield different results. The supporting evidence for the contribution of ROS to TNFα-induced NF-κB activation comes mainly from the fact that antioxidants, such as *N*-acetyl-L-cysteine (NAC) or pyrrolidine dithiocarbamate (PDTC) block TNFα-induced nuclear translocation of NF-κB (Schreck and Baeuerle, 1991; Schreck et al, 1992). However, several lines of evidence argue the role of ROS in TNFα-induced NF-κB activation (Li and Karin, 1999; Bowie and O'Neill, 2000). First, the kinetics of nuclear translocation of NF-κB and ROS accumulation induced by TNFα appear to be different. IKK activity is rapidly induced after TNFα stimulation and usually occurs within 5 min, thus ROS, even if induced by TNFα under normal conditions, do not appear to mediate this rapid activation of IKK (Li and Karin, 1999; Bowie and O'Neill, 2000). Indeed, ROS accumulation by TNFα has been reported to occur at relatively later time points in the cells (Sakon et al, 2003). Secondly, the specificity of these inhibitors is still controversial. In this respect, Hayakawa et al. recently reported a clue to solve this long-lasting controversial problem (Hayakawa et al, 2003). By investigating the mechanisms by which NAC and PDTC inhibit TNFα-induced nuclear translocation of NF-κB, they found that in addition to their anti-oxidant function, NAC also inhibits the binding of TNFα to TNF receptor, whereas PDTC inhibits ubiquitin ligase activity responsible for IκB degradation. Furthermore, treatment of BHA, which is a more potent inhibitor than NAC, did not inhibit TNFα-induced nuclear translocation of NF-κB or degradation of IκB in HeLa cells as well as wild-type MEFs (**Figure 7**).
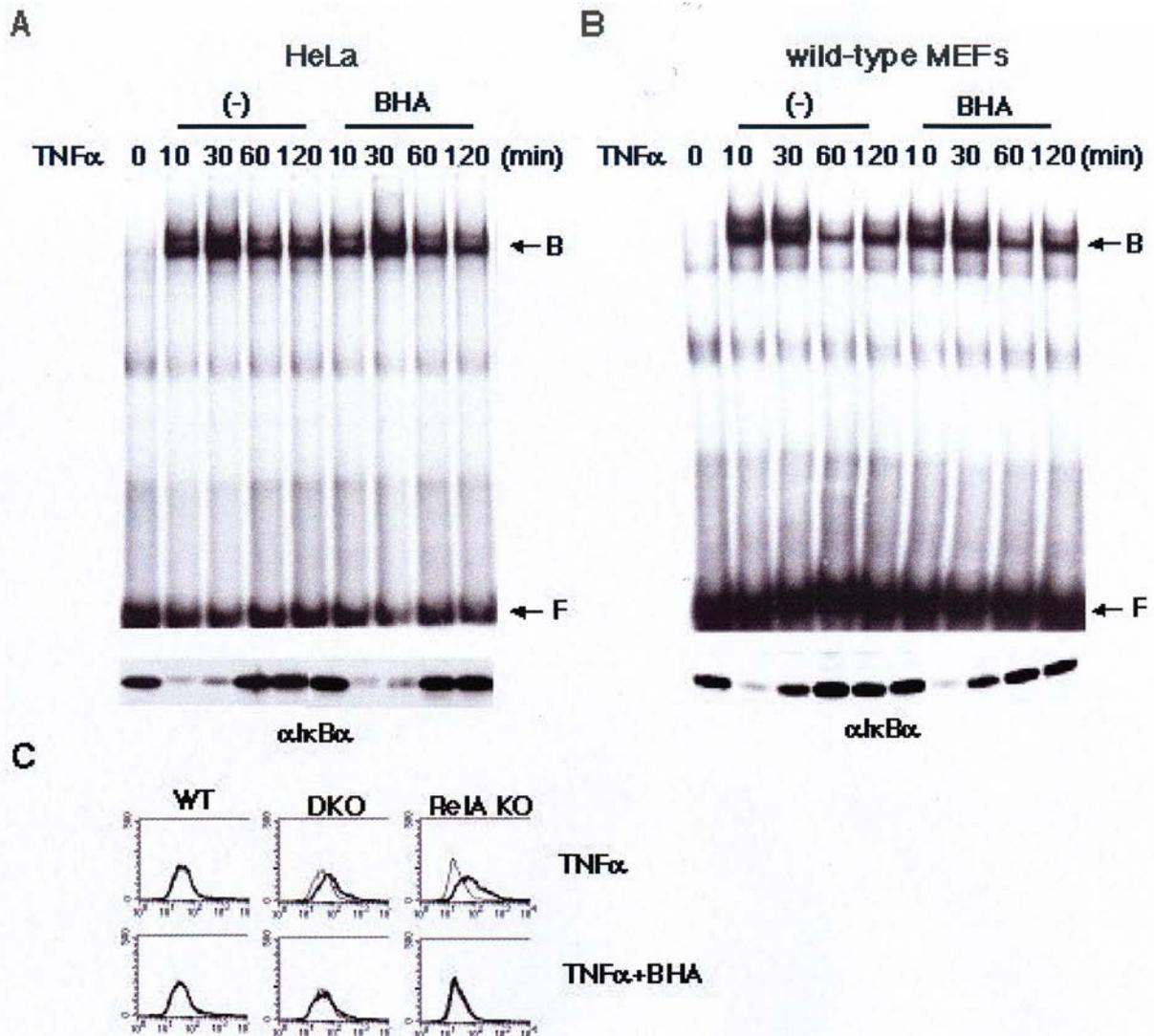
Figure 7. Antioxidant, BHA did not inhibit nuclear translocation of NF-κB or degradation of IκB, but inhibited TNFα-induced ROS accumulation. HeLa cells (**A**) or wild-type MEFs (**B**) were stimulated with human (**A**) or murine (**B**) TNFα (10 ng/ml) in the absence or presence of BHA (100 μM). After indicated time periods after stimulation, nuclear extracts and whole cell lysates were prepared. Nuclear extracts (upper panels) were subjected to electrophoretic gel mobility shift assay (EMSA) using ^{32}P-labeled NF-κB oligos. B and F indicate probes containing NF-κB complex and free probes, respectively. The whole cell lysates (lower panels) were blotted with anti-IκB antibody. (**C**) WT, DKO, and RelA KO MEFs were unstimulated (thin line) or stimulated (bold line) with TNFα (10 ng/ml) in the absence (upper panel) or presence of BHA (100 μM) (lower panel) for the indicated time periods, then the cells were labeled with CM-H$_2$DCFDA (1 μM) for the last 30 min, and analyzed by flow cytometry.

It is also still controversial whether exogenously added ROS induce nuclear translocation of NF-κB. Previous studies demonstrate that H$_2$O$_2$ can induce activation of NF-κB, but this activation is highly cell type specific (Bowie and O'Neill, 2000; Li and Karin, 1999). We also observed that H$_2$O$_2$ can induce nuclear translocation of NF-κB in HeLa cells (Figure 8A). However, such nuclear translocation of NF-κB is not induced in wild-type MEFs (Figure 8B). Importantly, the kinetics of activation was much slower than with TNFα

stimulation, but similar to those of genotoxic stress-induced NF-κB activation (Hur et al, 2003; Campbell et al, 2004), further substantiating that ROS do not contribute to TNFα-induced NF-κB activation. It is currently unknown whether H$_2$O$_2$-induced delayed nuclear translocation of NF-κB is dependent on IKK, or tyrosine phosphorylation of IκB as reported in an earlier study (Koong et al, 1994). In either case, these data indicate that ROS do not play major role in TNFα-induced NF-κB activation.
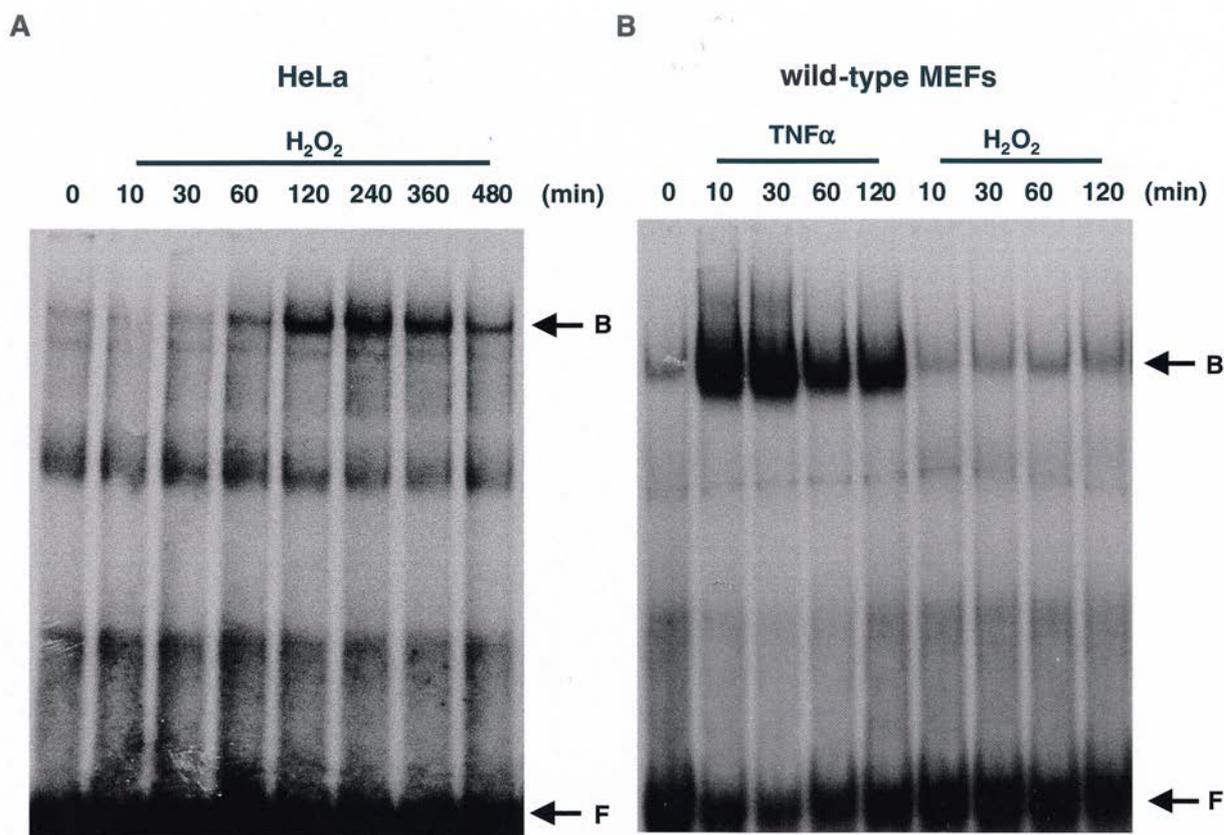
Figure 8. H_2O_2 induced nuclear translocation of NF- B in HeLa cells, but not wild-type MEFs. HeLa cells (**A**) or wild-type MEFs (**B**) were stimulated with H_2O_2 (1 mM) or murine TNF (10 ng/ml). After indicated time periods after stimulation, nuclear extracts were prepared and subjected to EMSA as described in Figure 7.

VI. How does TNFR signaling induce accumulation of ROS and cellular necrosis?

Under various pathological conditions such as ischemia, excessively accumulated ROS induce apoptosis or necrosis by activating MAPK, caspase cascades, and /or disruption of mitochondrial membrane potential (Fiers et al, 1999). Contribution of ROS to apoptosis and necrosis is highly cell-type specific. Given that ROS induce activation of JNK cascade, apoptosis induced by ROS is dependent on JNK-mediated mitochondrial dependent apoptotic pathway. Alternatively, ROS may directly act on mitochondria and induce the mitochondrial membrane permeabilization (MMP), resulting in release of apoptogenic factors, such as cytochrome c, apoptosis-inducing factor (AIF), and/or Smac/DIABLO (Kroemer and Reed, 2000; Wang, 2001). On the other hand, it is well known that TNF induces ROS-dependent necrosis in murine fibrosarcoma, L929 cells (Vercammen et al, 1998). Geldanamycin treatment induces degradation of HSP90 and its client protein, RIP, and shifted from necrosis to apoptosis in L929 cells, indicating an essential role of RIP in TNF -induced necrosis (Vanden Berghe et al, 2003). Holler et al. also reported that critical contribution of RIP to Fas-, TNF-, and TRAIL- induced necrotic cell death, although they did not investigate the contribution of ROS to these receptor-mediated necrosis, reaching a conclusion that FADD and kinase activity of RIP, but not caspase 8,

are essential for necrosis (Holler et al, 2000). Consistent with their results, preliminary experiments showed that ectopic expression of truncated FADD lacking death effector domain or protease inactive mutant of caspase 8 did not inhibit TNF -induced ROS accumulation and necrosis in DKO and RelA KO MEFs (Nakano et al, unpublished results), indicating that signaling pathways inducing necrosis and apoptosis might bifurcate downstream of FADD and upstream of caspase 8, perhaps implicating c-FLIP in this process. Very recently, Lin et al. reported similar findings that TNF induce ROS-dependent necrosis in the presence of broad caspase inhibitor, z-VAD-fmk, although the requirement of signaling intermediates for necrosis appears to be different from our study (Lin et al, 2004).

The mechanism by which ROS induces necrosis is still controversial. Several lines of evidence suggested that poly (ADP-ribose) polymerase (PARP) is activated by ROS, then depletes the NAD^+ pool by massive poly (ADP-ribosyl)ation (Los et al, 2002; Yu et al, 2002). In turn, NAD^+ depletion induces consumption of ATP, depriving the cells of its energy sources, culminating in death. This model appears to be very attractive due to the following reasons. First, under the conditions in which apoptosis is induced, PARP is cleaved by caspases and becomes inactivated not to induce necrosis. Secondly, pretreatment with a caspase inhibitor is required for efficient induction of necrosis under many experimental conditions, indicating that apoptotic pathway inhibits necrotic
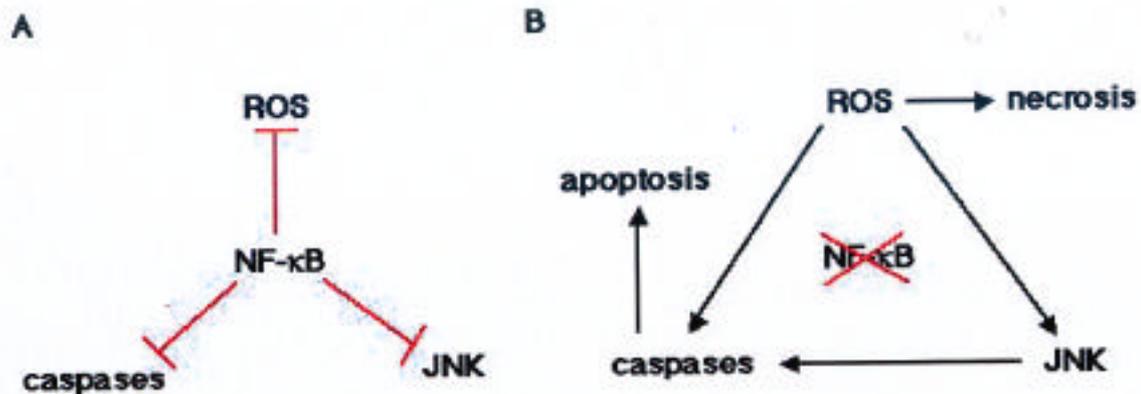
Figure 9. NF- B is a central regulator to determine cell death and cell survival.(**A**) Under normal conditions, NF- B inhibits three potentially apoptotic signaling cascades, including caspases, JNK, and ROS. (**B**) Under the pathological conditions, in which NF- B activation is blocked, three pro-apoptotic signaling cascades are activated and amplified by each other, resulting in apoptosis or necrosis in a context-dependent manner.

pathway by inactivating PARP. However, the PARP story does not appear to account for TNF -induced necrosis in DKO and RelA KO cells, since specific inhibitors for PARP, such as 1,5-dihydroxyisoquinoline (DHIQ) or 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ) did not inhibit TNF -induced necrosis (Nakano et al, unpublished results). Moreover, cellular ATP levels were not decreased in DKO and RelA KO cells after TNF stimulation (Nakano et al, unpublished results). These findings are also supported by studies from Lin et. al. (Lin et al, 2004). Taken together, the contribution of the PARP pathway to necrosis is cell-type specific, indicating that alternate routes to cellular necrosis may exist.

VII. Concluding remarks

Recent advances in gene targeting technique convincingly demonstrate the pro-apoptotic and anti-apoptotic function of JNK signaling cascade. Although the caspase cascade is sufficient for induction of apoptosis, the activation of JNK pathway itself does not appear to be sufficient for determining the cell fate. As Lin describes, JNK cascade modulates the pathways to cell death or survival (Lin, 2003). In this respect, the central regulator of determining the cell fate is NF- B. As described in **Figure 9A**, activation of NF- B is sufficient for inhibiting the cascades induced by pro-apoptosis-inducing molecules, caspases, JNK, and ROS in normal cells. Under the conditions, in which NF- B-mediated survival signals are blocked (such as cellular parasitism by viruses and other pathogens, or genotoxic stresses), JNK and ROS promote cell death in a context-dependent manner (**Figure 9B**). More importantly, several studies indicate that treatment of cells with caspase inhibitors rather enhances ROS-dependent necrosis *in vitro* and *in vivo* (Cauwels et al, 2003; Vercammen et al, 1998). To understand the NF- B-mediated survival signals in more detail and develop novel strategies to prevent excessive cell death under the pathological conditions, future studies will be focused on identifying molecules involved in JNK activation and ROS accumulation.

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