

Temporal pattern of NF κ B activation influences apoptotic cell fate in a stimuli-dependent fashion

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Summary

The transcription factor NF κ B is a critical immediate early response gene involved in modulating cellular responses and apoptosis following diverse environmental injuries. The activation of NF κ B is widely accepted to play an anti-apoptotic role in cellular responses to injury. Hence, enhancing NF κ B activation in the setting of injury has been proposed as one potential therapeutic approach to environmental injuries. To this end, we constructed a recombinant adenoviral vector (Ad.I κ B α AS) expressing antisense I κ B α mRNA that is capable of augmenting NF κ B activation prior to and following four types of cellular injury [TNF- α , UV, hypoxia/reoxygenation (H/R) or pervanadate treatment]. Biochemical and functional analyses of NF κ B activation pathways for these injuries demonstrated two categories involving either serine (S32/36) phosphorylation (TNF- α , UV) or tyrosine (Y42) phosphorylation (H/R or PV) of I κ B α . We hypothesized that activation of NF κ B prior to injury using antisense I κ B α mRNA would reduce apoptosis. As anticipated, recombinant adenoviral I κ B α phosphorylation mutants

(Ad.I κ B α S32/36A or Ad.I κ B α Y42F) preferentially reduced NF κ B activation and enhanced apoptosis following injuries associated with either serine or tyrosine phosphorylation of I κ B α , respectively. These studies demonstrate for the first time that an I κ B α Y42F mutant can effectively modulate NF κ B-mediated apoptosis in an injury-context-dependent manner. Interestingly, constitutive activation of NF κ B following Ad.I κ B α AS infection reduced apoptosis only following injuries associated with I κ B α Y42, but not S32/36, phosphorylation. These findings demonstrate that the temporal regulation of NF κ B and the apoptotic consequences of this activation are differentially influenced by the pathway mediating NF κ B activation. They also provide new insight into the therapeutic potential and limitations of modulating NF κ B for environmental injuries such as ischemia/reperfusion and pro-inflammatory diseases.

Key words: Antisense inhibition, NF κ B activation, Signal transduction, Apoptosis

Introduction

The NF κ B transcription factor family consists of five members called p50, p52, p65 (RelA), c-Rel and RelB, all of which can form various homo- and heterodimers (Karin and Ben-Neriah, 2000). NF κ B is normally sequestered in the cytoplasm by proteins of the I κ B family, I κ B α , β and γ . The predominant induced form of NF κ B, a p50 and p65 heterodimer, translocates to the nucleus upon activation. Common activating stimuli of the NF κ B pathway include interleukin-1 (IL-1), tumor necrosis factor (TNF- α), lipopolysaccharide (LPS), hypoxia/reoxygenation and phorbol myristate acetate (PMA) (Piette et al., 1997).

The most commonly studied pathway of NF κ B activation involves the phosphorylation of I κ B α on serine residues 32 and 36 by a recently identified I κ B kinase (IKK) complex, which is composed of three subunits, α , β and γ (Zandi et al., 1997). IKK α and IKK β are the two catalytic subunits in the complex. Phosphorylation of I κ B by the IKK complex leads to ubiquitination and degradation of I κ B, which unmasks a nuclear targeting sequence on the NF κ B molecule. This promotes the translocation of NF κ B from the cytoplasm to the nucleus where it becomes an active transcription factor

(Scherer et al., 1995). Regulators of IKK activity include NF κ B-inducing kinase (NIK), MEKK1 and NF κ B-activating kinase (NAK). These regulators impart signal-specific activation of NF κ B through the IKK complex. TNF- α and IL-1 induction of NF κ B is mediated by NIK phosphorylation of IKK α (Hirano et al., 1996). By contrast, MEKK1, which is part of the Jun N-terminal kinase/stress-activated protein kinase pathway, can induce the activation of both IKK α and IKK β (Lee et al., 1997). Most recently, NAK has been shown to directly phosphorylate IKK β (Tojima et al., 2000).

An alternative, but less studied mechanism for NF κ B activation, involves tyrosine phosphorylation of I κ B α leading to dissociation of NF κ B from I κ B α without proteolytic degradation (Fan et al., 1999). It was discovered that stimulation of Jurkat T-cells with either pervanadate (a protein phosphatase inhibitor) or hypoxia/reoxygenation leads to phosphorylation of I κ B α at tyrosine residue 42 and subsequent NF κ B nuclear translocation without I κ B proteolysis (Imbert et al., 1996). Similarly, in a neuron cell model, nerve growth factor treatment leads to NF κ B activation through I κ B α tyrosine phosphorylation independent of proteolytic degradation (Bui et al., 2001). In support of this mechanism,

our group recently demonstrated that NF κ B is activated without concomitant degradation of I κ B in a murine model of liver ischemia/reperfusion (I/R) (Zwacka et al., 1998). Furthermore, tyrosine phosphorylation of I κ B α was increased following I/R injury in the liver, suggesting that this degradation-independent pathway of NF κ B activation is important in I/R injury (Zwacka et al., 1998). Although these previous studies conclusively demonstrate that Y42 of I κ B α is phosphorylated following hypoxia/reoxygenation, the functional importance of this phosphorylation event in regulating NF κ B transcriptional activation has yet to be proven using a dominant-negative I κ B α Y42 phosphorylation mutant. In the case of both I/R and hypoxia, these findings have implicated a potentially unique tyrosine kinase in the phosphorylation of I κ B α . Furthermore, this kinase appears to be distinct from those activated by TNF- α , IL-1, LPS or PMA. The mechanism that leads to I κ B α tyrosine phosphorylation and NF κ B activation remains unclear; nonetheless, several key players have been identified. C-src, an osteoclast regulatory protein, has been shown to associate with I κ B α and phosphorylate I κ B α on tyrosine 42 in murine bone marrow macrophages (BMMs) in a cytokine-specific manner (Abu-Amer et al., 1998). Also, p85, the regulatory subunit of PI 3-kinase, specifically associates with tyrosine-phosphorylated I κ B α through its Src homology domains both in vitro and in vivo after stimulation of T cells with pervanadate (Beraud et al., 1999). These studies suggest that PI 3-kinase might be a candidate for regulating tyrosine phosphorylation of I κ B α .

As a pro-inflammatory transcription factor, NF κ B activation in the initial phase of I/R injury may trigger upregulation of cytokines, including TNF- α and IL-1, and adhesion molecules, such as ICAM-1, which can mediate the ensuing subacute inflammatory response (Fan et al., 1999). However, in addition to its pro-inflammatory action, NF κ B also plays a protective role in acute cellular stress responses that inhibit apoptosis following TNF- α treatment (Liu et al., 1996) or ionizing irradiation (Wang et al., 1996). Furthermore, it has been shown in a two-thirds hepatectomy model that inhibition of NF κ B by overexpression of a dominant-negative mutant form of I κ B α increases apoptosis and liver dysfunction (Iimuro et al., 1998). It is currently unclear how the detrimental proinflammatory and the beneficial anti-apoptotic effects of NF κ B activation are regulated in the setting of various types of injury.

In an effort to design therapeutic approaches to enhance NF κ B activation and reduce apoptosis following cellular injuries such as ischemia/reperfusion, we have generated and characterized a novel adenoviral vector that expresses an antisense mRNA of I κ B α and enhances NF κ B activation. Since two distinct pathways of regulating NF κ B through I κ B α serine or tyrosine phosphorylation have been identified, we have compared how altering the temporal expression profile of NF κ B influences apoptosis following four types of injury that utilize either serine or tyrosine I κ B α kinase activation pathways. Three recombinant adenoviral vectors expressing a serine mutant (Ad.I κ B α S32/36A), tyrosine mutant (Ad.I κ B α Y42F) or antisense mRNA (Ad.I κ B α AS) of I κ B α were used to modulate NF κ B activation prior to injury, and the apoptotic outcomes were compared. Results from these studies suggest that the timing of NF κ B activation during the acute phases of injury can significantly influence apoptotic outcomes

in an injury-stimuli-dependent manner. Interestingly, activating NF κ B prior to injury was anti-apoptotic only following stimuli dependent on tyrosine kinase activation of I κ B α but not IKK-dependent serine phosphorylation of I κ B α . These findings suggest that the temporal regulation of NF κ B following injury is an important feature that influences apoptotic cell fate in a manner which is also dependent on the pathway of NF κ B activation.

Materials and Methods

Construction of recombinant adenoviruses

The I κ B α (Y42F) cDNA was a kind gift from J. F. Peyron (Pasteur Institute, France). The plasmid was digested with *Hind*III to generate a fragment containing the entire coding region for the human I κ B α cDNA. This *Hind*III fragment was bidirectionally cloned into the recombinant adenovirus proviral plasmid, pAd.CMV link, which contains the cytomegalovirus (CMV) enhancer/promoter and an SV40 poly-adenylation site for efficient expression of the transgenes (Davis and Wilson, 1996). Two proviral plasmids containing the sense and antisense orientation of the I κ B α (Y42F) cDNA were generated, pAd.I κ B α (Y42F) and pAd.I κ B α AS, respectively. Recombinant viruses were then generated by co-transfection of an *Nhe*I-cut pAd plasmid, with *Cl*aI-cut Ad5.GFP viral DNA. Following co-transfection, plates were overlaid with agar, and initial plaques were harvested for screening by Southern blotting. Clones containing cDNAs were replaques two times, and viral stocks were purified by standard protocols (Davis and Wilson, 1996). The Ad.I κ B α (S32/36A) dominant-negative mutant adenoviral construct was a kind gift from David Brenner (UNC) (Iimuro et al., 1998).

Cell culture, adenoviral infection and environmental injury

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 μ g/ml penicillin and streptomycin. HeLa cells were infected with various recombinant adenoviral constructs at a multiplicity of infection (moi) ranging from 1000 to 1500 particles/cell. Adenoviral infections were performed for 2 hours in DMEM without FBS, followed by the addition of an equal volume of DMEM with 20% FBS. Fresh media was applied to cells when virus was removed at 24 hours post-infection. Experiments were typically performed at 24-72 hours post-infection. Experimental stimuli used to induce NF κ B were performed by the protocols listed below.

UV irradiation

HeLa cells were washed with PBS three times and irradiated with UV-C at 50 J/m² in the absence of surface liquid. Fresh medium was quickly applied to the plates following irradiation. Cells were harvested 2 hours post-irradiation for EMSA and 6 hours post-irradiation for NF κ B activation luciferase assays. Control, mock-irradiated cells were treated in a similar fashion but were not exposed to UV.

TNF- α treatment

Human recombinant TNF- α (R&D systems, Minneapolis, MN) was diluted to 10 ng/ml in fresh medium just prior to use. TNF- α -containing medium was applied to HeLa cells at the time of stimulation and cells remained exposed to TNF- α until they were harvested for analysis. Cells were harvested at 1 hour post-TNF- α treatment for EMSA and 6 hours post-TNF- α treatment for NF κ B activation luciferase assays. Control cells were fed with fresh medium without TNF- α .

Pervanadate(PV) treatment

500 mM sodium orthovanadate was prepared fresh in water. 40 μ l of sodium orthovanadate was then added to a mixture of 450 μ l PBS and 10 μ l 30% (w/w) H₂O₂. This solution was incubated for 5 minutes at room temperature prior to the addition of catalase (200 μ g/ml final concentration). The final pervanadate solution (40 mM) was incubated for 5 minutes at room temperature to remove excess H₂O₂ and then immediately diluted in DMEM for application to cells. Cells were continuously exposed to pervanadate containing medium until cells were harvested for analysis. Harvesting took place at 2 hours post-pervanadate treatment for EMSA and at 6 hours post-pervanadate treatment for NF κ B activation luciferase assays. Control cells were fed fresh medium without pervanadate.

Hypoxia/reoxygenation (H/R) experiments

DMEM (no glucose, 0%FBS, 1%P/S) equilibrated in 5% CO₂/95% N₂ or 5% CO₂/95% O₂ was used as hypoxia medium and reoxygenation medium, respectively. HeLa cells were incubated with hypoxia medium in an airtight chamber equilibrated with 5% CO₂/95% N₂ at 37°C for 5 hours. After hypoxia was performed, the medium was replaced with reoxygenation medium and cells were incubated in a chamber flushed with 5% CO₂/95% O₂ at 37°C. Cells were harvested 3 hours after reoxygenation for EMSA and 6 hours after reoxygenation for NF κ B activation luciferase assays. Control cells were fed with fresh medium at the appropriate times but were exposed to 5% CO₂ in atmospheric oxygen.

Western blotting and electrophoretic mobility shift assays (EMSA)

Cytoplasmic and nuclear extracts were prepared by standard protocols (Andrews and Faller, 1991) and used for western blotting and EMSA, respectively. Briefly, 2 \times 10⁶ HeLa cells were collected into 1.5 ml centrifuge tubes by blunt scraping with 1 ml PBS. Cells were pelleted for 1 minute and were then resuspended in 400 μ l of cold buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride). Samples were incubated on ice for 10 minutes, then vortexed for 10 seconds and finally pelleted by brief centrifugation (1 minute). The supernatant was saved as the cytoplasmic extract for western blotting, and the pellet was resuspended in 100 μ l of storage buffer (20 mM HEPES, pH 7.9, 25% Glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.21 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride). Samples were then incubated on ice for another 20 minutes followed by centrifugation. Supernatants were collected for use as nuclear extracts. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). 5 μ g of cytoplasmic extracts were resolved on a 10% SDS-PAGE and then transferred to nitrocellulose membranes. Following standard protocols (Zwacka et al., 1998), I κ B α protein levels were determined by western blotting using monoclonal anti-I κ B α , anti-I κ B β and anti-actin antibodies (Santa Cruz Biotech, Santa Cruz, California). EMSA analysis and supershift assays were performed using an NF κ B-specific oligonucleotide (Promega, Madison, WI). The sequence was as follows: 5'-AGTTGAGGGGACTTTCCAGGC-3'. The double-stranded nucleotides were end-labeled with γ ³²P-ATP using T4 polynucleotide kinase. 5 μ g of nuclear extract was used in each assay for NF κ B DNA binding using standard protocols (Zwacka et al., 1998). NF κ B antibodies used for supershift EMSA (anti-p50, anti-p52, anti-c-Rel, anti-RelB and anti-p65) were purchased from Santa Cruz Biotech.

Apoptosis assays

HeLa cells were infected with either Ad.BgIII or Ad.I κ B α AS for 24 hours at an moi of 1000 particles/cell. Both groups were treated with

UV, TNF- α , pervanadate and hypoxia/reoxygenation. In the TNF- α treated group, HeLa cells were pretreated with proteasome inhibitor LLnL (40 μ M final concentration) (Calbiochem, La Jolla, California) for 30 minutes prior to treatment to enhance apoptosis. In all experimental groups, cells were trypsinized 18 hours following treatment and stained with annexin V-FITC and propidium iodide (PI) for apoptotic analysis. Annexin-V-FITC binding was performed using a kit from Pharmingen (Palo Alto, CA) according to the manufacturer's protocol. Briefly, 1 \times 10⁶ cells were washed twice with cold PBS and then resuspended in 1 ml 1 \times binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). 5 μ l of Annexin-V-FITC (100 μ g/ml) and 10 μ l of propidium iodide (50 μ g/ml) were then added to 100 μ l cell suspensions. Cells were incubated for 15 minutes in darkness at room temperature. This was followed by the addition of 400 μ l of 1 \times binding buffer. Samples were then analyzed by FACS. Apoptotic cells were identified as an annexin-V-positive population. The percentage of apoptotic cells was calculated as the fraction of annexin-V-positive/PI-positive cells.

Luciferase indicator assays for NF κ B transcriptional activation

NF κ B transcriptional activity was assessed using an Ad.NF κ BLuc reporter vector. This construct contains the luciferase gene driven by four tandem copies of the NF κ B consensus sequence fused to a TATA-like promoter from the Herpes simplex virus thymidine kinase gene (Sanlioglu et al., 2001). HeLa cells were infected with Ad.NF κ BLuc 24 hours prior to treatment by UV, TNF- α , pervanadate or H/R. 5 μ g of total protein from each sample was used to perform luciferase assays. Luciferase activity was measured using a kit from Promega (Catalog No. E4030, Madison, WI).

I κ B α phosphorylation assays

Tyrosine phosphorylation of I κ B α was evaluated by immunoprecipitation from 200 μ g cytoplasmic extracts in 300 μ l RIPA buffer using 2 μ g I κ B α antibody (Santa Cruz Biotech, Santa Cruz, CA) overnight at 4°C. 30 μ l of Protein-A agarose was then added and incubated for 4 hours at 4°C. The agarose beads were then washed four times with ice-cold RIPA buffer before they were resuspended in SDS-PAGE loading buffer and loaded onto a 10% SDS-PAGE for western blot analysis. The membrane was probed with an antibody that recognizes phospho-tyrosine residues (Santa Cruz) at a dilution of 1:1000 following the western protocol outlined above. Serine phosphorylation of I κ B α was detected by standard western blotting of 5 μ g cell lysate using a phosphospecific antibody (Santa Cruz Biotech, Santa Cruz, California), which recognized phospho-S32/S36 of I κ B α .

Results

UV and TNF- α induce serine phosphorylation of I κ B α , whereas pervanadate and H/R induce tyrosine phosphorylation

Phosphorylation of I κ B α is a key step in the translocation of NF κ B to the nucleus. Phosphorylation of I κ B α on serine residues 32 and 36 by the inhibitor κ B kinase (IKK) complex leads to ubiquitination and degradation of I κ B proteins following pro-inflammatory stimuli. By contrast, tyrosine phosphorylation of I κ B α has been associated with the translocation of NF κ B to the nucleus without I κ B α proteolytic degradation following I/R. With the goal of modulating NF κ B activity to enhance cell survival following I/R injury, we sought to develop model systems capable of modulating NF κ B activity through tyrosine phosphorylation of I κ B α . Two such *in vitro* models including hypoxia/reoxygenation (H/R) and

pervanadate treatment have been shown to activate NF κ B in T-cells with a correlated increase in Y42 phosphorylation of I κ B α . In an effort to establish such a model for selective induction of NF κ B mediated by Y42 phosphorylation of I κ B α , we felt it would be useful to establish a cell model system also capable of inducing NF κ B activation through the better characterized IKK-dependent S32/36 phosphorylation of I κ B α . To this end, many cell lines were screened for their ability to induce NF κ B activation following four types of environmental injury, including UV, TNF- α , pervanadate and H/R treatments, which are known to involve either serine or tyrosine phosphorylation of I κ B α . HeLa cells were chosen for their ability to induce NF κ B DNA binding following each of these stimuli.

To experimentally establish that HeLa cells were capable of modulating NF κ B through these two independent serine or tyrosine I κ B α kinase pathways, we evaluated the state of I κ B α serine and tyrosine phosphorylation following the four types of environmental injury. Using a phosphospecific antibody that specifically recognizes the serine-phosphorylated form of I κ B α (S32/36), western blot analysis demonstrated that both UV and TNF- α treatments induced substantial levels of I κ B α serine phosphorylation by 15 to 30 minutes (Fig. 1A). By contrast, no significant change in the baseline levels of serine phosphorylation was observed following pervanadate or H/R treatment. Using immunoprecipitation of I κ B α followed by western blotting with an anti-phosphotyrosine antibody, we addressed the tyrosine-phosphorylated state of I κ B α . Results from these studies demonstrated that I κ B α tyrosine phosphorylation significantly increased at 15-30 minutes following pervanadate or H/R treatment (Fig. 1B). By contrast, no increase in tyrosine phosphorylation of I κ B α was detected following UV or TNF- α treatment. In summary, this data demonstrates that HeLa cells can be used as a cell model to study NF κ B activation mediated by either serine or tyrosine phosphorylation of I κ B α .

I κ B α (S32/36A) and I κ B α (Y42F) mutants selectively inhibit NF κ B activation in an injury-stimulus-dependent fashion

Although Y42 phosphorylation of I κ B α has been associated

with NF κ B activation following pervanadate and H/R treatments, functional evidence for the importance of this tyrosine phosphorylation event in the transcriptional activation of NF κ B using dominant-negative mutants of I κ B α is still lacking. To this end, we generated a recombinant adenovirus encoding the I κ B α (Y42F) mutant and evaluated its ability to inhibit transcriptional activation of an NF κ B-responsive luciferase reporter (also delivered via a recombinant adenovirus vector) following each of the various types of injury. As an important control for IKK-mediated pathways of NF κ B activation, a similar adenovirus vector encoding a dominant-negative I κ B α (S32/36A) mutant (Iimuro et al., 1998) was also evaluated. Consistent with the finding that I κ B α serine phosphorylation was most significantly induced by TNF- α and UV, I κ B α (S32/36A) expression significantly inhibited NF κ B transcriptional activation following TNF- α (98.7 \pm 5.21%, P <0.001) and UV (74.2 \pm 9.1%, P <0.001) treatments (Fig. 2). By contrast, I κ B α (S32/36A) expression did not significantly inhibit NF κ B activation following pervanadate (P =0.104) or H/R treatment (P =0.464) (Fig. 2). These data confirm that the predominant pathway controlling both TNF- α and UV induction of NF κ B in HeLa cells occurs through serine phosphorylation of I κ B α . Furthermore, they also demonstrate that serine phosphorylation of I κ B α plays only a minor role in regulating NF κ B transcriptional activity following pervanadate and H/R stimuli.

Using our newly constructed recombinant adenoviral vector expressing the Y42F mutant of I κ B α , we next sought to confirm that tyrosine phosphorylation of I κ B α was predominantly responsible for the transcriptional activation of NF κ B following pervanadate or H/R treatment. Consistent with the tyrosine phosphorylation data, I κ B α (Y42F) expression significantly inhibited NF κ B transcriptional activation following pervanadate (71.6 \pm 6.3%, P <0.01) and H/R (73.7 \pm 10.7%, P <0.01) treatments (Fig. 3). By contrast, both TNF- α and UV induction of NF κ B transcriptional activity was not significantly altered by expression of I κ B α (Y42F) (P =0.359 and P =0.257, respectively). In summary, these data demonstrate that the I κ B α (Y42F) mutant can selectively inhibit NF κ B transcriptional activation following pervanadate or H/R treatment. They also confirm that tyrosine

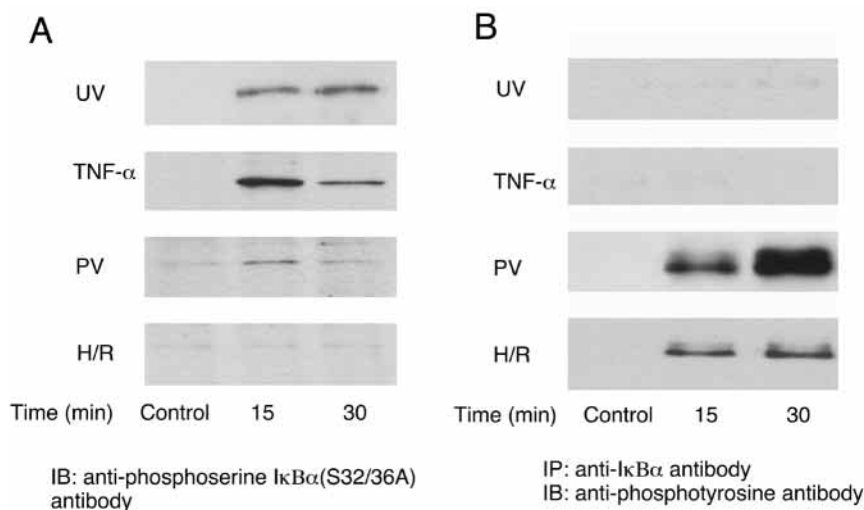


Fig. 1. TNF- α and UV treatment lead to serine phosphorylation of I κ B α , whereas pervanadate and H/R induce tyrosine phosphorylation of I κ B α . HeLa cells were treated with UV (50 J/m²), TNF- α (10 ng/ml), pervanadate (50 μ M) or H/R (5 hours hypoxia, 15 and 30 minutes reoxygenation) for the indicated times. Both untreated and treated samples were harvested for cytoplasmic extracts. (A) 5 μ g of total protein was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Phosphorylation of I κ B α at S32/36 was evaluated by a phosphospecific antibody. (B) 200 μ g of total cytoplasmic protein was immunoprecipitated with anti-I κ B α antibody followed by western blotting with an anti-phosphotyrosine antibody to detect tyrosine phosphorylation of I κ B α . Samples analyzed were identical to those evaluated in A.

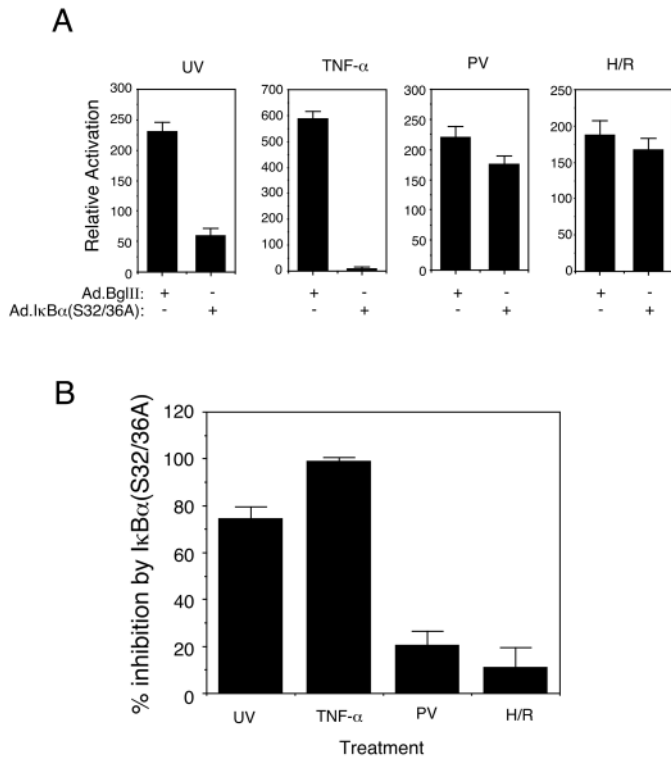


Fig. 2. IκBα(S32/36A) inhibits NFκB activation following UV and TNF-α treatment. (A) HeLa cells were co-infected with Ad.IκBα(S32/36A) or Ad.BglIII together with Ad.NFκBLuc 24 hours before treatment. Cells were harvested 6 hours after UV (50 J/m²), TNF-α (10 ng/ml), pervanadate (50 μM) and H/R (5 hours hypoxia, 6 hours reoxygenation) treatments, and whole cell extracts were normalized by total protein content and subjected to luciferase assays. NFκB activity was determined by the relative luciferase activity (as light units). The relative NFκB activation was calculated by deducting the mean NFκB baseline activation for each vector group (in the absence of stimulation) from the corresponding individual values from the same vector group in the presence of stimulus. The relative NFκB activation is plotted for each individual stimulus (±s.e.m., n=6). (B) Percent NFκB inhibition by IκBα(S32/36A) for each experimental point was calculated using the following formula: percent inhibition=1-(relative activation of each Ad.IκBα(S32/36A) infected sample/mean relative activation of the Ad.BglIII infected group). Results depict the mean (±s.e.m.) for n=6 independent data points in each group.

phosphorylation of IκBα is the predominant mechanism of NFκB transcriptional activation by these stimuli.

Inhibition of NFκB activation through either IκBα tyrosine or serine phosphorylation pathways stimulates apoptosis following environmental injuries

Although it is widely accepted that activation of NFκB following cellular injury is anti-apoptotic, this phenomena has not been established for injuries that activate NFκB via tyrosine phosphorylation of IκBα. The lack of information on this topic is probably due to the lack of efficient vectors capable of expressing high levels of a IκBα(Y42F) mutant in the majority of cells. However, since recombinant adenoviral vectors are capable of expressing high levels of IκBα mutants,

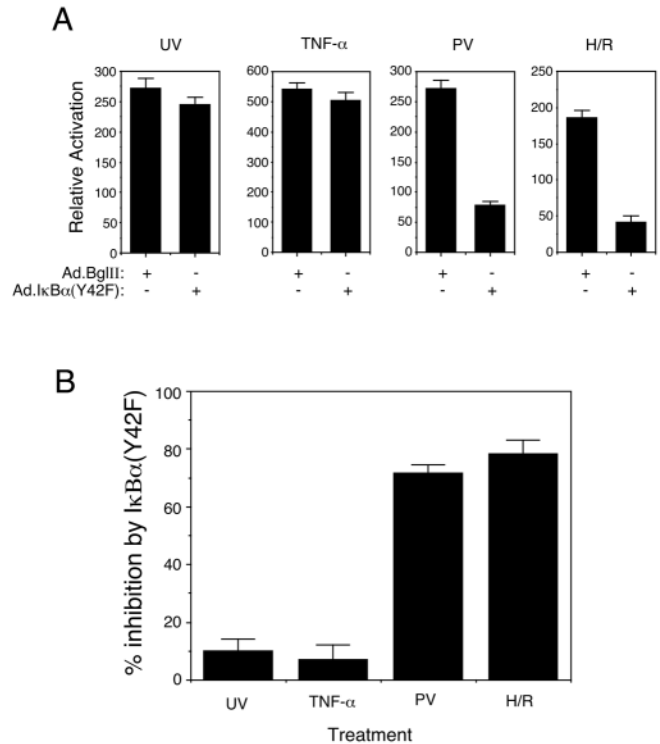


Fig. 3. IκBα(Y42F) inhibits NFκB activation following pervanadate and H/R treatment. (A) HeLa cells were co-infected with Ad.IκBα(Y42F) or Ad.BglIII together with Ad.NFκBLuc 24 hours before treatment. Cells were treated with UV (50 J/m²), TNF-α (10 ng/ml), pervanadate (50 μM) and H/R (5 hours hypoxia, 6 hours reoxygenation) then harvested 6 hours after treatment. Whole cell extracts were normalized by total protein content and subjected to luciferase assay. NFκB activity was determined by the relative luciferase activity (as light units). The relative NFκB activation was calculated by deducting the mean NFκB baseline activation for each vector group (in the absence of stimulation) from the corresponding individual values from the same vector group in the presence of stimulus. The relative NFκB activation is plotted for each individual stimulus (±s.e.m., n=6). (B) Percent NFκB inhibition by IκBα(Y42F) for each experimental point was calculated using the following formula: percent inhibition=1-(relative activation of each Ad.IκBα(Y42F) infected sample/mean relative activation of the Ad.BglIII infected group). Results depict the mean (±s.e.m.) for n=6 independent data points in each group.

we sought to conclusively address this issue by comparing the ability of either IκBα(S32/36A) or IκBα(Y42F) mutant to selectively enhance apoptosis following each of the previously described types of environmental injury (Fig. 4). Results from these experiments demonstrated that expression of the IκBα(S32/36A) mutant preferentially enhanced apoptosis following TNF-α or UV treatment to a far greater extent than that seen following expression of the IκBα(Y42F) mutant ($P<0.02$). Thus inhibiting NFκB activation with Ad.IκBα(S32/36A) has a stronger influence on apoptosis following UV or TNF-α treatment when compared to Ad.IκBα(Y42F). By contrast, expression of the IκBα(Y42F) mutant preferentially enhanced apoptosis following H/R or pervanadate treatment to a greater extent than that seen following expression of the IκBα(S32/36A) mutant ($P<0.03$).

The inhibition of NF κ B activation with Ad.I κ B α (Y42F) had a stronger influence on apoptosis following pervanadate or H/R treatment compared with Ad.I κ B α (S32/36A). Although this differential effect of tyrosine and serine mutants of I κ B α was not absolute, these results do functionally substantiate findings for two independent pathways of NF κ B activation. More importantly, they also demonstrate that inhibition of NF κ B activation stimulates apoptosis regardless of the injury pathway responsible for NF κ B activation.

Antisense inhibition of I κ B α protein enhances NF κ B baseline activation

The anti-apoptotic nature of NF κ B suggests that augmenting NF κ B activation prior to injury might enhance the protective effects of this molecule. Indeed, such therapeutic strategies appear to be the basis for protective effects following ischemic preconditioning in the heart (Morgan et al., 1999). To this end, we sought to evaluate whether inhibition of I κ B α protein synthesis using an antisense mRNA approach could reduce apoptosis and improve cell survival following the various types of environmental injuries. A recombinant adenovirus (Ad.I κ B α AS) encoding I κ B α cDNA in the reversed orientation was tested for its ability to inhibit I κ B α protein expression and enhance NF κ B activation. As seen in Fig. 5A, infection of HeLa cells with Ad.I κ B α AS

significantly reduced steady-state levels of I κ B α protein. Interestingly the steady-state level of I κ B β was increased in the presence of antisense I κ B α mRNA. This probably reflects a compensatory mechanism induced by activation of NF κ B. These findings also demonstrated specificity of the antisense approach to inhibit I κ B α expression. Despite the observed changes in both I κ B α and I κ B β levels, no changes in the steady-state level of the actin internal control were observed. Ad.I κ B α AS infection consequently led to a time-dependent increase in nuclear NF κ B DNA binding that was not observed in Ad.GFP-infected controls (Fig. 5B). EMSA supershift assay using antibodies to various NF κ B subunits (p50, p52, c-Rel, RelB, and p65) demonstrated that I κ B α AS expression induced NF κ B complexes composed of p50/p65 heterodimers (Fig. 5C). These results demonstrate that inhibition of I κ B α protein expression using an antisense mRNA approach significantly elevates baseline levels of NF κ B in the nucleus.

Having demonstrated that infection with Ad.I κ B α AS enhances baseline NF κ B DNA-binding activity in the nucleus by inhibiting I κ B α protein expression, we next sought to confirm that expression of I κ B α AS mRNA enhanced NF κ B transcriptional activation following UV, TNF- α , pervanadate or H/R treatment. In these studies, HeLa cells were infected with either a control adenovirus, Ad.BglIII, or Ad.I κ B α AS at an moi of 1000 particles/cell 24 hours prior to exposure to UV, TNF- α , pervanadate or H/R. As previously reported, each of these treatments induced NF κ B DNA binding (Fig. 6); however, the time course of NF κ B activation varied. In uninfected cells, NF κ B DNA binding peaked 2 hours following UV irradiation or pervanadate treatment, 1 hour following TNF- α treatment and 3 hours following H/R treatment (data not shown). Hence, for comparative studies of Ad.BglIII- and Ad.I κ B α AS-infected groups, samples were harvested at the peak time points of NF κ B DNA binding following a given stimulus. Results from this analysis clearly demonstrated that infection with Ad.I κ B α AS increased nuclear NF κ B DNA binding following UV (Fig. 6A), TNF- α (Fig. 6B), pervanadate (Fig. 6C) or H/R (Fig. 6D) treatment. In summary, these results demonstrate that inhibiting I κ B α protein expression enhances activation of NF κ B DNA binding in the nucleus following exposure to all types of stimuli tested.

Since DNA binding is only one indicator of NF κ B activation, we also sought to directly evaluate the transcriptional activity of NF κ B using the recombinant adenoviral reporter vector (Ad.NF κ BLuc) harboring a NF κ B-inducible luciferase gene. In these studies, HeLa cells were co-infected with Ad.NF κ BLuc and Ad.I κ B α AS or Ad.BglIII 24 hours prior to treatments. Whole cell extracts were harvested 6 hours following treatment and luciferase activity was measured as an indicator of NF κ B transcription activation. Consistent with our EMSA result on NF κ B DNA binding, NF κ B transcriptional activity was also significantly enhanced ($P < 0.01$) in the Ad.I κ B α AS-infected groups, as compared to the groups infected by Ad.BglIII, following each of the environmental injuries (Fig. 7).

Activation of NF κ B prior to injury is pro-apoptotic following UV or TNF- α treatment but anti-apoptotic following pervanadate or H/R treatment

On the basis of our findings that dominant-negative

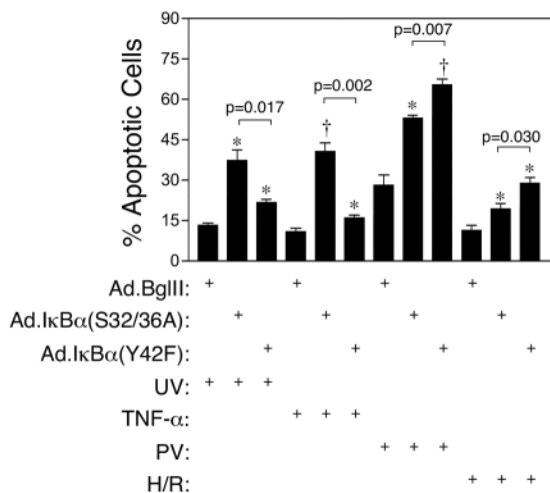


Fig. 4. Inhibition of either tyrosine or serine I κ B α phosphorylation stimulates apoptosis in an injury-context specific fashion. HeLa cells were infected with Ad.BglIII, Ad.I κ B α (S32/36A) or Ad.I κ B α (Y42F) at an moi of 1000 particles/cell for 24 hours. Cells were treated with UV (50 J/m²), TNF- α (10 ng/ml), pervanadate (50 μ M) or H/R (5 hours hypoxia, 18 hours reoxygenation) and then harvested 18 hours after treatment. Cells were then stained with annexin-V-FITC and propidium iodide. Results depict the mean (\pm s.e.m., $n=3$) percent of apoptotic cells as shown by FACS analysis. Ad.I κ B α (S32/36A) more significantly increased apoptosis following UV or TNF- α treatment, whereas Ad.I κ B α (Y42F) preferentially increased apoptosis following pervanadate or H/R treatment. Paired t -test analysis was performed between Ad.BglIII infected and Ad.I κ B α (S32/36A) or Ad.I κ B α (Y42F) infected samples for each stimulus, and a statistically significant difference is denoted by * ($P < 0.05$) or † ($P < 0.001$). P -values for paired t -test analysis comparing Ad.I κ B α (S32/36A) and Ad.I κ B α (Y42F) infected samples for each stimulus are denoted above brackets.

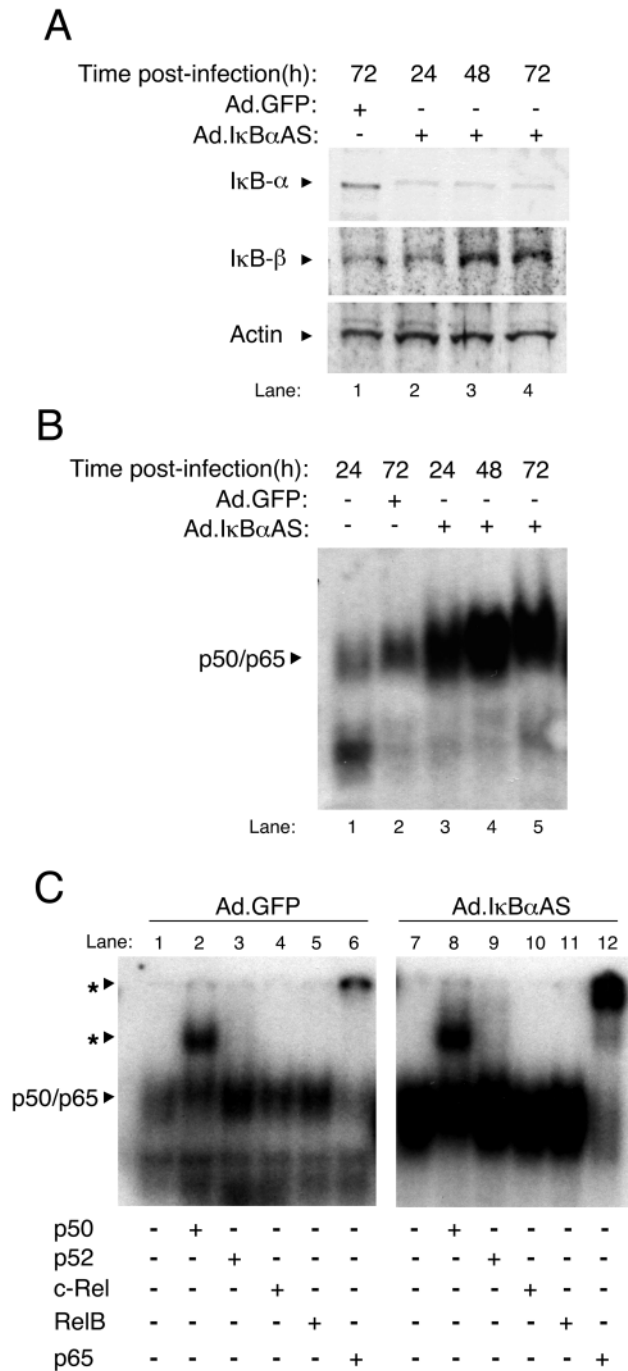


Fig. 5. Ad.IκBαAS inhibits IκBα protein expression and elevates baseline levels of NFκB in the nucleus. HeLa cells were infected at an moi of 1000 particles/cell with Ad.IκBαAS or Ad.GFP. Cytoplasmic and nuclear extracts were prepared from cells collected at 24, 48 and 72 hours after infection. (A) 5 μg of cytoplasmic protein was evaluated by western blotting using anti-IκBα antibody, anti-IκBβ antibody or anti-actin antibody. Ad.IκBαAS infection led to a reduction in IκBα protein expression at all time points (lane 2-4) and an increase in IκBβ expression compared with Ad.GFP-infected controls (lane 1). Changes in IκB expression were referenced to actin protein levels, which did not change. (B) 5 μg of nuclear protein was evaluated by EMSA to determine NFκB DNA-binding activity. The induced p65-p50 heterodimer of NFκB, identified by supershift assays, is marked by an arrow. Results in A and B are derived from the same experimental samples, and experimental conditions are marked above each lane. (C) 5 μg of nuclear protein from 72 hour post-infection time points with Ad.GFP (same sample as from lane 2 Panel B) and Ad.IκBαAS (same sample as from lane 5 Panel B) were evaluated by EMSA supershift to determine the subunit composition of the NFκB DNA binding complex. Antibodies used for supershift were against p50, p52, C-Rel, Rel B and p65. The active DNA-binding complex is identified as p65-p50 heterodimers, and supershifted bands (in lanes 2, 6, 8 and 12) are marked by an asterisk to the left of the gel.

infected cells ($8.9\% \pm 0.5$) as compared to the Ad.BglIII control group ($4.6\% \pm 0.3$). In stark contrast, following PV treatment, IκBαAS expression significantly decreased ($P < 0.01$) the percentage of apoptotic cells to $16.0\% \pm 1.5$ compared with the Ad.BglIII-infected control group ($30.4\% \pm 2.0$). Similarly, following H/R, the Ad.IκBαAS-infected group demonstrated a significantly lower ($P < 0.01$) apoptotic rate ($4.0\% \pm 0.5$) as compared with the control group ($6.0\% \pm 0.7$). These findings demonstrate that increased NFκB activation during the acute phase of injury can have either pro-apoptotic or anti-apoptotic effects depending on the type of injury stimulus. Interestingly, this differential apoptotic effect of enhancing NFκB activation prior to injury correlated with either serine or tyrosine IκBα phosphorylation pathway responsible for the activation of NFκB following injury. Enhancing NFκB activity prior to injury was pro-apoptotic for stimuli that promote IKK-mediated serine phosphorylation of IκBα (TNF-α and UV), although it was anti-apoptotic for stimuli that promote tyrosine phosphorylation of IκBα (H/R and pervanadate). Although these findings suggest that enhancing NFκB activity may be a viable strategy for reducing apoptosis following I/R injury, they also suggest that the temporal regulation of NFκB is an important component in determining its apoptotic influence for other types of injuries.

phosphorylation mutants of IκBα preferentially enhanced apoptosis following each of the environmental injuries tested, we hypothesized that elevating the level of NFκB activation with IκBαAS prior to injury would have the reverse effect. Interestingly, results from these experiments demonstrated two distinct functional consequences of IκBαAS expression depending on the type of environmental injury evaluated (Fig. 8). Following UV treatment, IκBαAS expression significantly ($P < 0.001$) increased apoptosis to $40.6\% \pm 2.2$ as compared to the Ad.BglIII-infected control group ($15.5\% \pm 0.8$). Similar observations were seen in TNF-α treated cells, which had a two-fold increase ($P < 0.01$) in apoptosis in Ad.IκBαAS-

Discussion

The manner in which NFκB balances both the detrimental pro-inflammatory and beneficial anti-apoptotic consequences of its activation in a way that promotes cell survival following injury remains a paradox. Additionally, alternative pathways for activating NFκB in response to injury that are independent of IKK-mediated serine phosphorylation of IκBα and ubiquitin-dependent degradation are being increasingly appreciated. This alternative pathway of activating NFκB through tyrosine phosphorylation of IκBα appears to be critical following I/R injury. The goals of this study were designed to evaluate

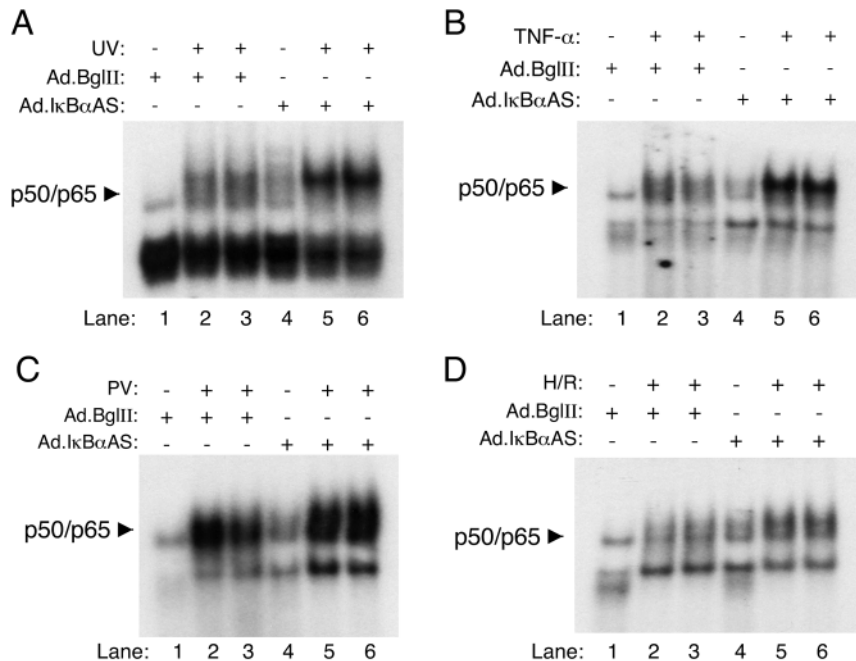


Fig. 6. IκBαAS enhances NFκB binding activity following UV, TNF-α, pervanadate or H/R treatment. HeLa cells were infected with either Ad.BgIII or Ad.IκBαAS at an moi of 1000 particles/cell for 24 hours. Cells were treated with (A) UV (50 J/m²), (B) TNF-α (10 ng/ml), (C) pervanadate (50 μM) or (D) H/R (5 hours hypoxia, 6 hours reoxygenation). Both treated and untreated cells were collected for nuclear extract preparation and EMSA analysis. Ad.IκBαAS-infected cells demonstrated a higher baseline of NFκB DNA-binding activity (lanes 1 versus lane 4) and enhanced NFκB activation following all treatments compared with Ad BgIII-infected cells (lanes 2,3 versus lanes 5,6). The conditions for various experimental groups are marked above each panel.

potential therapeutic strategies to enhance NFκB activation and increase cell survival following I/R injury using in vitro H/R as a model. The importance of IκBα tyrosine phosphorylation in NFκB transcriptional activation and cell survival following I/R injury has never been directly addressed in the literature owing to the lack of highly transducible vector systems expressing the IκBα(Y42F) mutant. Given the diversity of pathways associated with NFκB activation, we also felt that comparing injury stimuli that activate NFκB through either serine or tyrosine phosphorylation of IκBα would also be very useful in evaluating the consequences of modulating NFκB activity on apoptosis. Important to this comparison was the establishment of a HeLa cell line model capable of responding to diverse injury stimuli that activate NFκB through these two

independent pathways. Such a model system in a single cell line assures that differences in the pathways of NFκB activation are due to differences in the injury stimuli and not cell-specific differences in the abundance of signal transduction intermediates.

Studies establishing this model system confirmed that both UV and TNF-α treatment leads to serine phosphorylation of IκBα. By contrast, hypoxia/reoxygenation and pervanadate (a tyrosine phosphatase inhibitor) treatment led to tyrosine phosphorylation of IκBα. Although these studies are confirmatory in nature, they do establish that this model system specifically modulates IκBα serine or tyrosine phosphorylation in an injury-specific manner within the same cell type. The functional specificity of either IκBα S32/36 or Y42

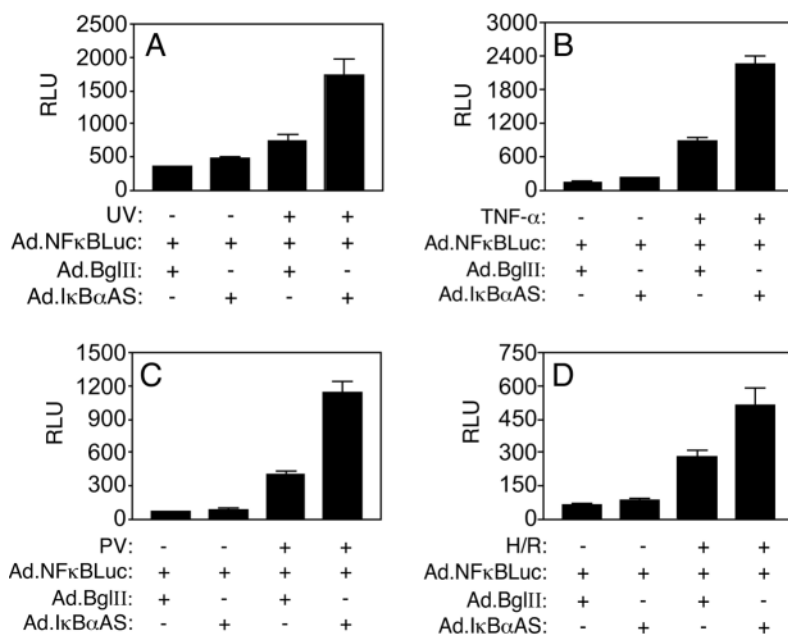


Fig. 7. IκBαAS enhances NFκB transcriptional activity following UV, TNF-α, pervanadate or H/R treatment. HeLa cells were co-infected with Ad.NFκBLuc (500 particles/cell) together with either Ad.BgIII or Ad.IκBαAS at an moi of 1000 particles/cell for 24 hours. Cells were treated with (A) UV (50 J/m²), (B) TNF-α (10 ng/ml), (C) pervanadate (50 μM) and (D) H/R (5 hours hypoxia, 6 hours reoxygenation). Luciferase assays were performed on samples harvested 6 hours after treatment. Results depict the mean (±s.e.m., n=6) raw unadjusted relative light units (RLU). NFκB transcriptional activity was significantly enhanced (*P*<0.01) in the Ad.IκBαAS infected groups, as compared to the groups infected by Ad.BgIII, following UV, TNF-α, pervanadate or H/R treatment.

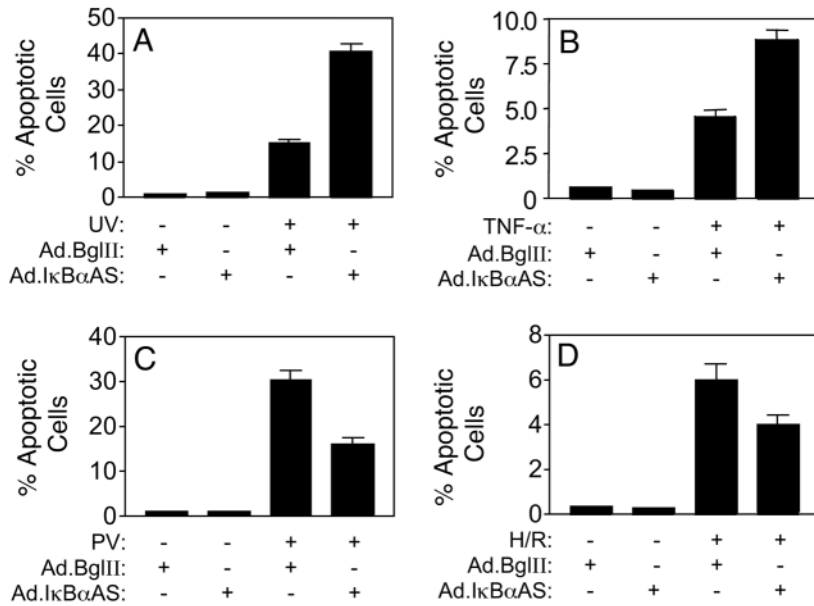


Fig. 8. NF κ B activation is pro-apoptotic following UV and TNF- α treatments, while anti-apoptotic following pervanadate and H/R treatments. HeLa cells were infected with either Ad.BgIII or Ad.I κ B α AS at an MOI of 1000 particles/cell for 24 hours. Cells were treated with (A) UV (50 J/m²), (B) TNF- α (10 ng/ml), (C) pervanadate (50 μ M) and (D) H/R (5 hours hypoxia, 18 hours reoxygenation) then harvested 18 hours after treatment. Cells are then trypsinized and stained with annexin-V-FITC and propidium iodide. Results depict the mean (\pm s.e.m., $n=3$) percent of apoptotic cells as shown by FACS analysis. Following UV and TNF- α treatment, I κ B α AS expression significantly ($P<0.01$) increased apoptosis compared with the Ad.BgIII-infected control group. By contrast, following pervanadate and H/R treatments, I κ B α AS expression significantly decreased ($P<0.01$) the percentage of apoptotic cells as compared to the Ad.BgIII-infected control group.

phosphorylation in promoting NF κ B activation in response to specific injury stimuli was also confirmed using transcriptional reporter assays and dominant-negative Y42F or S32/36A mutants of I κ B α . These studies for the first time demonstrate that Y42, but not S32/36, phosphorylation of I κ B α is the predominant pathway for transcriptional activation of NF κ B following H/R in an epithelial cell line model.

Although the present study has tried to distinguish between serine and tyrosine phosphorylation of I κ B α following each environmental injury, it is recognized that overlap in these two activation pathways may exist. For example, it is still debated whether UV irradiation activates NF κ B through IKK-mediated I κ B α serine phosphorylation. Several reports have demonstrated that the dominant mutants IKK α (KM) or I κ B α (S32/36A) can inhibit UV-induced NF κ B activation (Kulms et al., 2000; Li et al., 2001). However, others have reported that NF κ B activation following UV-C irradiation does not involve the IKK complex or I κ B α serine phosphorylation (Bender et al., 1998; Li and Karin, 1998). Differences in these results may be cell line dependent. Although most recent results demonstrate that the majority of UV-induced NF κ B activation can be inhibited by I κ B α (S32/36A), the extent of inhibition (74%) was much less than that seen for TNF- α (99%). Furthermore, 10% of UV-induced NF κ B transcriptional activity was inhibited by the I κ B α (Y42F) mutant, and although this inhibition was not statistically significant, it does suggest that some overlap in these two pathways may exist. Alternatively, additional non-I κ B α -associated mechanisms controlling NF κ B activation, such as Akt phosphorylation of p65 (Madrid et al., 2000), may also play some minor role in these studies.

Controversies regarding the involvement of NF κ B in both anti-apoptotic and pro-apoptotic pathways have been difficult to reconcile (Lipton, 1997). In part, the complexity and diversity of pathways that can activate NF κ B has fed this controversy. Importantly, determinants of cell survival and apoptosis are, themselves, complex and not solely regulated by a single factor such as NF κ B. Hence, independent pathways of

NF κ B activation may impart unique functional consequences to NF κ B activity by altering the composition of signaling intermediates that may act in concert to facilitate or prevent apoptosis under a given stimulus. The functional consequences of inhibiting NF κ B following pro-inflammatory stimuli such as TNF- α have been well documented to enhance apoptosis in the presence of I κ B α (S32/36A) expression. However, similar information is lacking for H/R injury using a I κ B α (Y42F) mutant. Important to designing therapeutic strategies for preventing apoptosis following I/R by modulating NF κ B activity was to first establish whether its inhibition of NF κ B was pro- or anti-apoptotic in the setting of H/R. Studies using either I κ B α (S32/36A) or I κ B α (Y42F) mutants confirmed that inhibiting NF κ B under all environmental stimuli was pro-apoptotic. Although each of these mutants preferentially enhanced apoptosis following injuries specific for their respective pathways of I κ B α phosphorylation, some overlap was seen. For example, although I κ B α (Y42F) expression more significantly increased apoptosis following H/R than I κ B α (S32/36A), the serine mutant also significantly enhanced apoptosis in comparison with Ad.BgIII-infected controls. This overlap was much higher than that seen in studies evaluating the specificity of these mutants to transcriptionally activate NF κ B following each of the independent stimuli. We reason that the difference in the specificity of I κ B α mutants to modulate NF κ B transcriptional activation or apoptosis is due to the longer time course required to achieve apoptosis in the setting of injury. Hence, although the acute stages of NF κ B activation may be selective for serine or tyrosine I κ B α phosphorylation, during the later stages of injury greater overlap in these two pathways may exist owing to activation of NF κ B-responsive cytokines that restimulate cells through alternative NF κ B pathways. Nonetheless, these studies conclusively demonstrate for the first time that inhibition of NF κ B activation using an I κ B α (Y42F) mutant enhances apoptosis following H/R.

Having demonstrated that activation of NF κ B is anti-apoptotic following H/R, we hypothesized that enhancing

NF κ B activation prior to injury might provide a protective advantage to cells. In support of this hypothesis, infection with recombinant adenovirus expressing antisense I κ B α mRNA significantly enhanced NF κ B transcriptional activation and reduced apoptosis following either H/R or PV treatment. Surprisingly, similar studies in TNF- α - or UV-treated cells gave rise to the opposite result. Although expression of antisense I κ B α mRNA similarly enhanced NF κ B transcriptional activation following either TNF- α or UV treatment, a significant increase in apoptosis was observed with these stimuli in antisense I κ B α -mRNA-expressing cells. Hence, elevation of NF κ B transcriptional activity prior to injury promoted apoptosis following TNF- α or UV treatment, although it inhibited apoptosis following pervanadate or H/R treatment. These findings suggest that the temporal regulation of NF κ B influences apoptosis in a stimuli-dependent fashion.

One interesting feature of the differential effects of enhancing NF κ B on apoptosis following these various stimuli, is the fact that they fell into two distinct groups associated with a particular pathway for NF κ B activation. For example, both UV- and TNF- α -treated groups, which demonstrated enhanced apoptosis in the setting of elevated NF κ B activation, utilize S32/36 phosphorylation of I κ B α as the pathway for NF κ B activation. By contrast, both H/R- and pervanadate-treated groups, which demonstrated reduced apoptosis in the setting of elevated NF κ B activation, utilize Y42 phosphorylation of I κ B α as the pathway for NF κ B activation. These findings demonstrate that, depending on the type of environmental injury and the pathway for NF κ B activation, the consequences of enhancing NF κ B activation prior to injury can produce dramatically different phenotypes in relation to programmed cell death. We hypothesize that this previously unidentified relationship between IKK or protein tyrosine kinase (PTK) pathways of NF κ B activation, and the ultimate apoptotic consequences of this activation, is probably due to additional stimuli-specific factors induced under each of the given conditions. NF κ B most probably works in concert with such factors to determine apoptotic cell fates. This current hypothesis would propose that activated NF κ B following IKK or PTK stimuli is biochemically identical and that stimuli-specific factors linked to either IKK or PTK pathways of NF κ B activation uniquely alter the subset of injury response genes transcriptionally activated by NF κ B complexes and/or the consequences of that activation. However, it is interesting that dominant-negative phosphorylation mutants of I κ B α enhanced apoptosis following all of the environmental stimuli tested. These findings suggest that NF κ B can indeed play an anti-apoptotic role during the later phases of injury. Taken together, results from experiments using antisense and dominant-negative mutants of I κ B α suggest that the temporal regulation of NF κ B at the time of injury is an important component in determining cell fate.

Evidence for such diversity in NF κ B involvement in apoptosis is supported by findings in the literature (Baichwal and Baeuerle, 1997; Jobin and Sartor, 2000; Yamamoto and Gaynor, 2001). For example, in neuronal cells, NF κ B activation mediates glutamate-induced toxicity and subsequent cell death and blocking NF κ B activation with aspirin or salicylate protects cells from the neurotoxic effects of glutamate (Grilli et al., 1996). Similarly, in a study of serum-starvation-induced apoptosis, a dominant-negative mutant of

the NF κ B p65 subunit has been shown to suppress transcription activity of NF κ B and partially inhibit apoptosis in a human embryonic kidney cell line (293 cells) (Grimm et al., 1996). In contrast to these studies, inhibition of NF κ B activation by expression of a recombinant, dominant-negative serine mutant of I κ B α (S32/36A) leads to massive apoptosis in the liver following partial hepatectomy (Iimuro et al., 1998). Similar findings in IKK knockout mice also suggest that NF κ B plays an important role as an anti-apoptotic factor during liver development (Rudolph et al., 2000; Tanaka et al., 1999). Since β -catenin has also been shown to be a substrate of the IKK complex (Lamberti et al., 2001), we also evaluated whether expression of our various I κ B α constructs could affect β -catenin levels by substrate competition with the IKK complex and in turn affect the apoptotic outcome. However, our result demonstrated no significant change in β -catenin levels following infection with Ad.I κ B α AS, Ad.I κ B α (S32/36A) or Ad.I κ B α (Y42F) (data not shown).

From a therapeutic standpoint, the present studies suggest that strategies aimed at enhancing NF κ B activation to increase cell survival may be effective for only certain classes of injury stimuli (i.e. I/R) that activate NF κ B through I κ B α tyrosine kinase pathways. Such findings demonstrate that the pathway of NF κ B activation and the temporal regulation of its activation significantly influences apoptotic outcomes. In support of this concept, a recent study by Lawrence et al. reported that NF κ B activation during the immediate early and late phases of inflammation plays independent pro-inflammatory and anti-inflammatory roles in gene expression, respectively (Lawrence et al., 2001). Our studies demonstrating that the apoptotic outcomes of two independent pathways of NF κ B activation are differentially influenced by temporal alteration in the NF κ B activity support the notion of context-dependent roles for NF κ B during acute and late phase responses.

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