

Nuclear Factor- κ B (NF- κ B) Inhibitory Protein I κ B β Determines Apoptotic Cell Death following Exposure to Oxidative Stress^{*[S]}

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Background: The role of individual members of the I κ B family of inhibitory proteins in mediating oxidant stress-induced NF- κ B activity is unknown.

Results: I κ B β degradation occurs with oxidative stress, along with loss of basal NF- κ B activity, pro-apoptotic gene expression, and apoptosis.

Conclusion: Preventing oxidative stress-induced NF- κ B signaling through I κ B β prevents apoptosis.

Significance: Modulating I κ B β expression during oxidative stress represents a novel therapeutic target to limit cellular injury.

The transcription factor NF- κ B regulates the cellular response to inflammatory and oxidant stress. Although many studies have evaluated NF- κ B activity following exposure to oxidative stress, the role of the I κ B family of inhibitory proteins in modulating this activity remains unclear. Specifically, the function of I κ B β in mediating the cellular response to oxidative stress has not been evaluated. We hypothesized that blocking oxidative stress-induced NF- κ B signaling through I κ B β would prevent apoptotic cell death. Using I κ B β knock-in mice (AKBI), in which the I κ B α gene is replaced with the I κ B β cDNA, we show that I κ B β overexpression prevented oxidative stress-induced apoptotic cell death. This was associated with retention of NF- κ B subunits in the nucleus and maintenance of NF- κ B activity. Furthermore, the up-regulation of pro-apoptotic genes in WT murine embryonic fibroblasts (MEFs) exposed to serum starvation was abrogated in AKBI MEFs. Inhibition of apoptosis was observed in WT MEFs overexpressing I κ B β with simultaneous I κ B α knockdown, whereas I κ B β overexpression alone did not produce this effect. These findings represent a necessary but not sufficient role of I κ B β in preventing oxidant stress-induced cell death.

The transcription factor NF- κ B regulates the cellular response to inflammatory and oxidant stress. NF- κ B activation occurs via a well defined pathway following exposure to inflammatory stimuli (1). In contrast, the effect of oxidant stress on NF- κ B signaling is less clear. Studies have shown that oxidant stress can stimulate or antagonize NF- κ B activity in a cell type- and stimulus-specific manner (2). Furthermore, the signaling

pathway linking oxidative stress and NF- κ B signaling remains unclear (2).

In quiescent cells, NF- κ B remains sequestered in the cytoplasm bound to members of the I κ B family of inhibitory proteins (3). The principal I κ B isoforms maintaining NF- κ B complexes in the cytoplasm are I κ B α and I κ B β (4). Early studies to determine the specific role of I κ B α in NF- κ B signaling were complicated by perinatal lethality seen in I κ B α ^{-/-} mice (5, 6). To address this problem, Bravo and co-workers (7) generated mice in which I κ B β cDNA replaced the I κ B α gene. Studies of these I κ B β knock-in (or AKBI) mice suggested functional redundancy of I κ B α and I κ B β in regulating canonical NF- κ B activity using TNF- α as an inflammatory stimulus (7). However, recent investigations have begun to dissect the functional differences between I κ B α and I κ B β .

Exposure to inflammatory stimuli leads to phosphorylation of two N-terminal serine residues on both I κ B α and I κ B β , resulting in proteasomal degradation (8). Expression of I κ B α is transcriptionally regulated by NF- κ B, allowing for a sensitive and rapidly activated negative feedback loop that terminates NF- κ B activation (9). Newly synthesized I κ B α enters the nucleus and removes DNA-bound NF- κ B complexes (9–12). In contrast to I κ B α , I κ B β is degraded more slowly; it is not transcriptionally regulated by NF- κ B; and following degradation induced by inflammatory stimuli, it re-accumulates in a hypophosphorylated form that facilitates DNA binding of NF- κ B dimers (13–15). I κ B β has been shown to preferentially bind p65 and c-Rel NF- κ B subunits, but it does not bind p50 (16). This is important, as specific NF- κ B subunits demonstrate unique affinity for specific oligonucleotide sequences, thereby allowing for targeted regulation of gene expression (17).

A critical role of I κ B β in regulating the response to inflammatory stress has been demonstrated by recent publications using I κ B β ^{-/-} mice. Two separate groups demonstrated that I κ B β ^{-/-} mice were resistant to LPS-induced septic shock (18,

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19). These studies showed that I κ B β acted with p65-c-Rel complexes to increase the transcription of specific proinflammatory NF- κ B target genes (e.g. IL-1 β) and that I κ B β was responsible in part for fine-tuning the NF- κ B response to inflammatory stress. However, although multiple studies have investigated the role of I κ B β following exposure to inflammatory stress, its role in mediating the cellular response to oxidative stress remains unexplored.

Indirect evidence has emerged to suggest that I κ B β is important in the cellular response to oxidative stress. Mitochondrial stress releases NF- κ B dimers from I κ B β , resulting in nuclear translocation and up-regulation of specific target genes (20). Furthermore, Fan *et al.* (6) demonstrated that AKBI mice are resistant to ischemia-reperfusion injury of the liver; however, the role of I κ B β overexpression was not evaluated in this study. These studies suggest a stimulus-specific effect on NF- κ B signaling that proceeds through I κ B α or I κ B β .

Here, using serum starvation and glucose oxidase to induce oxidative stress, we show that I κ B β degradation stimulates a pro-apoptotic response. Furthermore, this effect is abrogated in AKBI murine embryonic fibroblasts (MEFs)² as well as WT MEFs with I κ B β overexpression. Thus, delaying activation of this pathway results in inhibition of apoptosis and confers resistance to the deleterious effects of serum starvation. These findings have implications for targeting therapies to modulate the cellular response to oxidative stress.

EXPERIMENTAL PROCEDURES

MEF Cell Culture—Wild-type (ICR) and AKBI MEFs were generated from embryos at embryonic day 12.5 after timed mating. Cells were immortalized by serial passage, and all experiments were performed after passage 25.

Cell Culture, Serum Starvation, Glucose Oxidase, and TNF- α Incubation—Both WT and AKBI MEF cells were grown in DMEM supplemented with 10% FBS, 1% antibiotic/antimycotic (Invitrogen), and 1% nonessential amino acid solution (Sigma) and maintained at 37 °V in 5% CO₂ and 95% room air. In all experiments, cells were seeded at 8500 cells/cm² in plastic culture dishes and allowed to adhere overnight prior to exposure. For serum starvation, the medium was aspirated from the cells (~80% confluent) and washed twice with PBS prior to exposure FBS-free (DMEM, 1% antibiotic/antimycotic, and 1% nonessential amino acid solution) medium. Exogenous oxidative stress was induced by glucose oxidase (1–10 milliunits/ml). Canonical NF- κ B activity was induced by TNF- α (10 ng/ml).

Preparation of Cell Lysate and Cytosolic and Nuclear Extractions—Cells were washed with ice-cold PBS (pH 7.4), trypsinized, and pelleted. Nuclear and cytosolic fractions were extracted using a nuclear protein extraction kit (Pierce) according to the manufacturer's instructions. Whole cell lysates were obtained using M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Protein content was determined by the Bradford method (Bio-Rad).

Cell Fractionation and Immunoblot Analysis—Cell lysate and cytoplasmic and nuclear extracts (20 μ g) were electrophoresed on a 4–12% polyacrylamide gel (Invitrogen). Proteins were transferred to an Immobilon membrane (Millipore). Membranes were blotted with anti-I κ B α (sc-371), anti-I κ B β (sc-9130), or anti-lamin B (sc-6216) antibody (Santa Cruz Biotechnology); anti-I κ B ϵ (9249), anti-I κ B kinase (IKK) α (2682), anti-IKK β (2370), anti-c-Rel (4774), anti-caspase-3 (9665), or anti-poly(ADP-ribose) polymerase (9542) antibody (Cell Signaling); anti-p50 antibody (Abcam ab7971); or anti-tubulin antibody (Millipore 05-829).

Evaluation of Cell Death Using Trypan Blue Exclusion—Trypan blue exclusion was used to determine cell viability. Cells were trypsinized, pelleted, resuspended in a 1:1 mixture of PBS and 0.5% trypan blue, and manually counted using a hemocytometer. The numbers of live (unstained) cells were then expressed as a ratio of the total (stained and unstained) cells counted.

Detection of Intracellular Reactive Oxygen Species—Cells were incubated with 10 μ M chloromethyl-H₂-2',7'-dichlorofluorescein diacetate (DCF-DA) (Invitrogen) for 15 min. Medium containing chloromethyl-H₂DCF-DA was aspirated, and cells were washed with PBS prior to exposures. Cell lysate (40 μ g) was loaded onto a 96-well plate, and fluorescence was measured (excitation wavelength of 492 nm and emission wavelength of 525 nm) using a SpectraMax M5 spectrophotometer and analyzed using SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

Evaluation of Caspase-3 and Caspase-9 Activities—Following exposure, cells were collected using the M-PER mammalian protein extraction reagent without protease inhibitors. Fluorometric caspase-3 (final concentration of 50 μ M; Upstate) or caspase-9 (Calbiochem) substrate was incubated with the cell lysate (30 μ g) for 60 min at 37 °C and evaluated following the manufacturer's instructions.

Caspase Inhibition—AKBI and ICR MEFs were pretreated with the irreversible caspase-3 inhibitor benzyloxycarbonyl-DEVD-fluoromethyl ketone (25 μ M; MBL International Corp., Woburn, MA) for 1 h prior to exposure to serum-free medium containing the inhibitor. Whole cell lysates were harvested and assessed for caspase-3 activity as described above.

Transfection of MEFs with NF- κ B Luciferase Reporter, I κ B Overexpression, and I κ B siRNA—MEF cells were grown to 70% confluence on a 6-well plate and transfected with the luciferase reporter gene (pNF- κ B-luc, Clontech) and with a *Renilla* luciferase plasmid (Promega), the pReceiver-m12 I κ B β expression plasmid (GeneCopoeia), or a pCMV-I κ B α vector (Clontech); and with I κ B α (5 nM) or I κ B β (5 nM) Silencer select siRNA (Ambion). MEF cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Eighteen hours after transfection, cells were exposed to serum-free medium (DMEM and 1% antibiotic/antimycotic) or TNF- α (10 ng/ml). Control and stimulated cells were then lysed, and luciferase activity was measured using the Dual-Luciferase assay kit (Promega) following the manufacturer's instructions.

² The abbreviations used are: MEF, murine embryonic fibroblast; IKK, I κ B kinase; DCF-DA, 2',7'-dichlorofluorescein diacetate.

I κ B β Determines Survival after Oxidative Stress

Determination of Apoptosis-related Gene Expression Profile by Targeted Microarray—The expression of genes involved in apoptosis was analyzed using the mouse apoptosis RT2 profiler PCR array (SABiosciences) according to the manufacturer's instructions. WT and AKBI MEFs were exposed to serum starvation for 4 h. Reverse transcription (RT2 first strand kit, SABiosciences) was performed on RNA (150 ng/sample, isolated using the RT2 qPCR-grade RNA isolation kit, SABiosciences). All conditions were run in triplicate, and data were analyzed using the web-based PCR array data analysis software (SABiosciences).

Statistical Analysis—For comparison between treatment groups, the null hypothesis that no difference existed between treatment means was tested by analysis of variance for multiple groups or by *t* test for two groups (InStat, GraphPad Software, Inc.). Statistical significance ($p < 0.05$) between and within groups was determined by the Bonferroni method of multiple comparisons.

RESULTS

AKBI MEFs Are Resistant to Oxidative Stress-induced Cell Death—To assess whether AKBI MEFs are more resistant to serum starvation-induced cell death, trypan blue exclusion assays were performed. No significant difference in the percentage of dead cells was found in cells grown in 10% serum (Fig. 1A). Following serum starvation for 16, 24, 48, and 72 h, ICR MEFs demonstrated 80, 75, 57, and 53% cell survival, respectively ($p < 0.05$ versus the control). In contrast, AKBI MEFs demonstrated no significant decrease in cell survival compared with control MEFs and significantly improved survival after 24, 48, and 72 h of serum starvation compared with similarly exposed WT MEFs ($p < 0.05$). Importantly, ICR and AKBI MEFs were equally sensitive to apoptosis induced by camptothecin (data not shown). This suggests that AKBI MEFs are specifically more resistant to serum starvation-induced cell death.

As serum starvation induces oxidative stress (21), the increased viability in AKBI MEFs could be explained by either enhanced antioxidant defense or unique signaling downstream of the oxidative stress. To determine whether serum starvation differentially induces oxidative stress in WT and AKBI MEFs, DCF-DA fluorescence was assessed. Following 2 h of serum starvation, both WT and AKBI MEFs demonstrated similar increases in DCF-DA fluorescence (Fig. 1B), suggesting that the observed differences in survival between the two cell lines are mediated by a differential response to the injury. Furthermore, changes in the protein levels of the NF- κ B-regulated antioxidants copper/zinc superoxide dismutase and manganese superoxide dismutase were evaluated in WT and AKBI MEFs exposed to serum starvation (supplemental Figs. S1 and S4). No difference in the protein levels was found in either cell line in response to serum starvation, suggesting that transcriptional regulation of antioxidants by NF- κ B does not explain differences seen between WT and AKBI MEFs.

To corroborate these findings, MEFs were exposed to glucose oxidase as an exogenous source of oxidative stress (22). Similar to the pattern of survival following serum starvation, WT MEFs demonstrated a time- and dose-dependent increase

in cell death after glucose oxidase exposure (Fig. 1, C and D). This effect was abrogated in AKBI MEFs (Fig. 1, C and D). Of note, increased reactive oxygen species formation was seen in both WT and AKBI MEFs exposed to glucose oxidase and hydrogen peroxide, consistent with the findings following serum starvation (Fig. 1, E and F, and supplemental Fig. S2). These results suggest that serum starvation, glucose oxidase, and hydrogen peroxide generate reactive oxygen species in both WT and AKBI MEFs.

Serum Starvation Induces Apoptosis in WT MEFs but Not in AKBI MEFs—To evaluate whether the increase in cell death following serum starvation is due to apoptosis, caspase-3 cleavage was assessed. A significant increase in WT MEF apoptosis following exposure to serum starvation was observed using Western analysis of cleaved caspase-3 (Fig. 2A). In contrast, serum starvation-induced caspase-3 cleavage was not observed in AKBI MEFs. Densitometric analysis confirmed a statistically significant increase in the ratio of cleaved caspase-3 to procaspase-3 in WT MEFs exposed to serum starvation, whereas no significant increase was seen in AKBI MEFs (Fig. 2B). Furthermore, a significant increase in caspase-3 activity was found in WT cells exposed to serum starvation for 6 h compared with controls (Fig. 2C). In contrast, there was no increase in caspase-3 activity in similarly exposed AKBI MEFs. Importantly, the increased caspase-3 activity seen in WT MEFs was completely abrogated by pretreating cells with the caspase-3 inhibitor benzyloxycarbonyl-DEVD-fluoromethyl ketone (Fig. 2C). Corroborating these findings, poly(ADP-ribose) polymerase cleavage was observed only in WT MEFs exposed to serum starvation (Fig. 2D and supplemental Fig. S4). Finally, to help determine whether oxidative stress initiates the intrinsic pathway of apoptosis, caspase-9 activity was assessed. This was significantly increased only in WT cells exposed to serum starvation (Fig. 2E). Together, these findings indicate that, in contrast to WT MEFs, AKBI MEFs are resistant to apoptosis induced by serum starvation.

Canonical NF- κ B Signaling Is Intact in AKBI MEFs—Altered expression of the I κ B family of proteins can affect expression of other NF- κ B family member proteins (23, 24). In AKBI MEFs, expression of p105, p65, p50 c-Rel, I κ B ϵ , IKK α , and IKK β was not different compared with WT cells as determined by Western blotting (Fig. 3A) or quantitative PCR (data not shown). As expected, AKBI MEFs had no I κ B α signal and overexpressed I κ B β compared with WT MEFs (Fig. 3A). TNF- α exposure was used to verify intact canonical NF- κ B signaling in AKBI MEFs (Fig. 3, B–F). Consistent with published data, TNF- α resulted in decreased I κ B α and I κ B β in WT MEFs after 15 and 60 min of exposure, respectively (Fig. 3, B and C). In AKBI MEFs, the pattern of I κ B β degradation mirrored that of I κ B α in WT MEFs, decreasing after 15 min of TNF- α exposure (Fig. 3C). Both WT and AKBI MEFs had significant increases in nuclear p65 following 15 min of TNF- α exposure (Fig. 3, D and E; purity of fractionation demonstrated in supplemental Fig. S3). Nuclear p65 persisted in AKBI MEFs for a longer duration compared with WT MEFs (2 versus 1 h) (Fig. 3, D and E). Despite this difference, NF- κ B luciferase reporter activity was not different between the two cell lines at 4 h of exposure (Fig. 3F). These results demonstrate intact and robust canonical NF- κ B

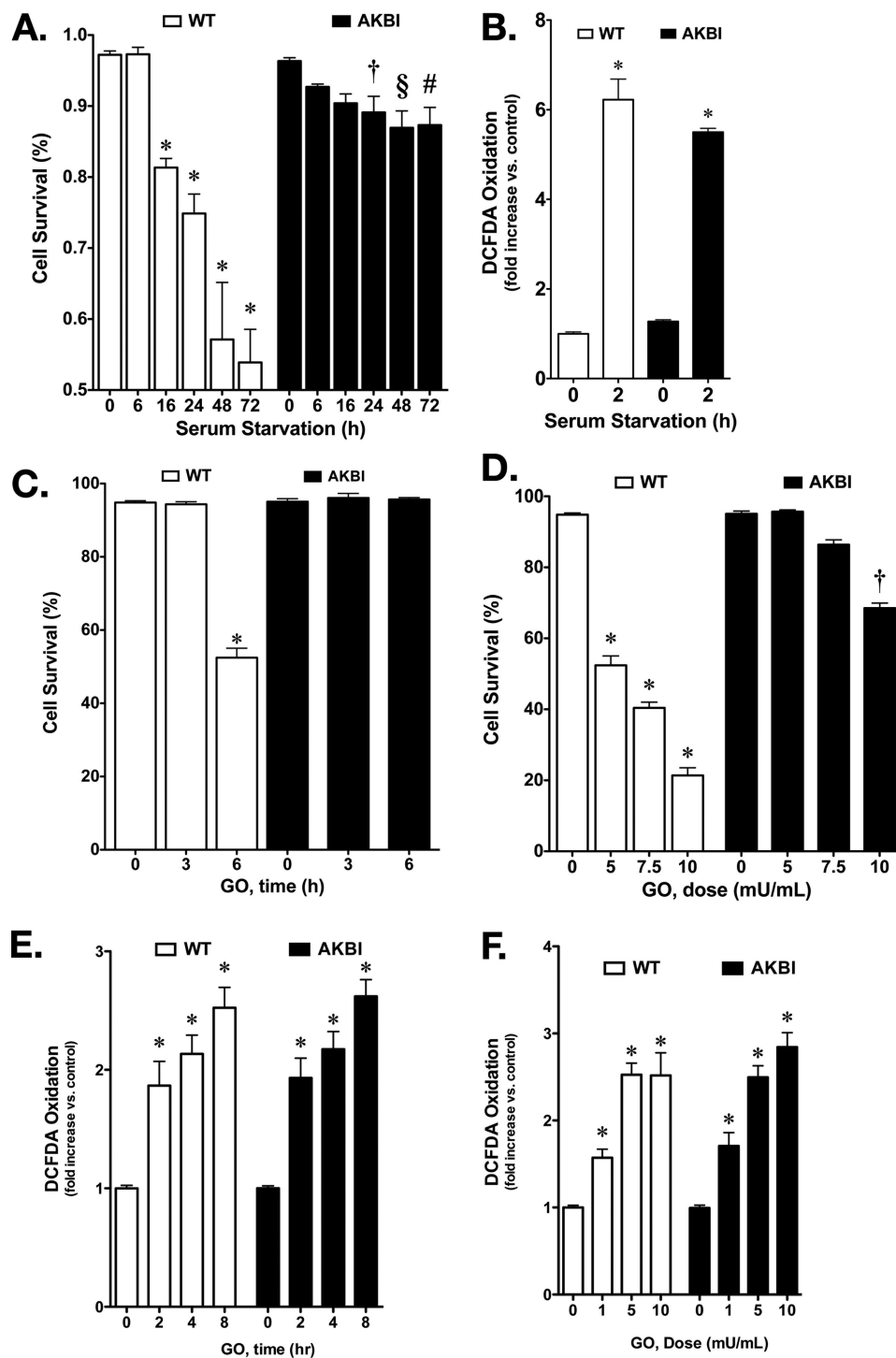


FIGURE 1. AKBI MEFs are resistant to serum starvation-induced cell death but not to reactive oxygen species production. *A*, WT and AKBI MEF cells were exposed to serum starvation. Viability was assessed by trypan blue exclusion. The graph represents percent viable cells after exposure. Values are means \pm S.E. of three independent experiments for each group. *, $p < 0.05$ versus the WT control; †, $p < 0.05$ versus WT at 24 h; §, $p < 0.05$ versus WT at 48 h; #, $p < 0.05$ versus the WT control and corresponding AKBI exposure. *B*, -fold induction of DCF-DA oxidation in cells exposed to serum starvation. Values are means \pm S.E. of three independent experiments for each group. *, $p < 0.05$ versus the respective control. *C*, WT and AKBI MEF cells were exposed to glucose oxidase (GO; 5 milliunits/ml). Viability was assessed by trypan blue exclusion. The graph represents percent viable cells after exposure. Values are means \pm S.E. of three independent experiments for each group. *, $p < 0.05$ versus the WT control and AKBI at 6 h. *D*, WT and AKBI MEF cells were exposed to glucose oxidase (5, 7.5, and 10 milliunits/ml) for 6 h. Viability was assessed by trypan blue exclusion. The graph represents percent viable cells after exposure. Values are means \pm S.E. of three independent experiments for each group. *, $p < 0.05$ versus the WT control and corresponding AKBI exposure; †, $p < 0.05$ versus the AKBI control. *E*, -fold induction of DCF-DA oxidation in cells exposed to glucose oxidase (5 milliunits/ml). Values are means \pm S.E. of three independent experiments for each group. *, $p < 0.05$ versus the respective control. *F*, -fold induction of DCF-DA oxidation in cells exposed to glucose oxidase (5, 7.5, and 10 milliunits/ml) for 6 h. Values are means \pm S.E. of three independent experiments for each group. *, $p < 0.05$ versus the respective control.

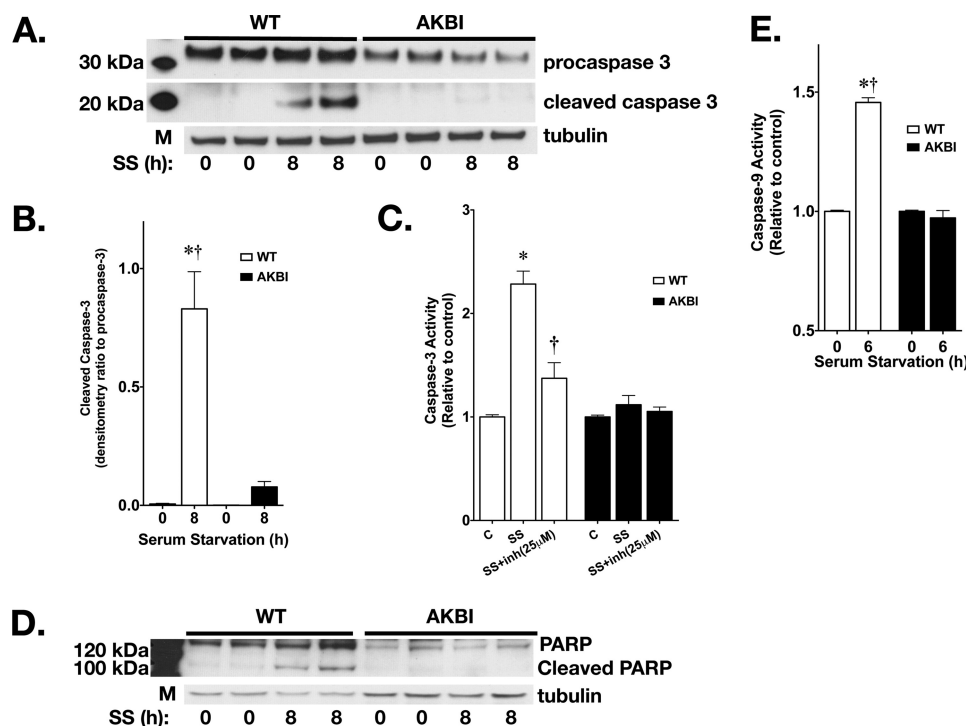


FIGURE 2. Apoptosis increases in WT MEFs exposed to oxidative stress. *A*, representative Western blot showing procaspase-3 and cleaved caspase-3 in WT and AKBI MEFs exposed to serum starvation (SS) with tubulin as a loading control. *M*, marker. *B*, densitometric evaluation of -fold increase in cleaved caspase-3 as a ratio to procaspase-3 from *A* after exposure to serum starvation. Values are means ± S.E. of three independent experiments for each group. **p* < 0.05 versus the WT control; †*p* < 0.05 versus AKBI at 8 h. *C*, the graph represents relative -fold caspase-3 activity compared with the respective controls following exposure to serum starvation. Values are means ± S.E. of three independent experiments for each group. *C*, control; *SS*, 6 h of serum starvation; *SS+inh*(25 μM), 6 h of serum starvation following a 1-h pretreatment with the caspase inhibitor benzyloxycarbonyl-DEVD-fluoromethyl ketone (25 μM). **p* < 0.05 versus the WT control and AKBI at 6 h; †*p* < 0.05 versus WT at 6 h. *D*, representative Western analysis showing poly(ADP-ribose) polymerase (*PARP*) and cleaved poly(ADP-ribose) polymerase in WT and AKBI MEFs exposed to serum starvation with tubulin as a loading control. *E*, the graph represents relative -fold increase in caspase-9 activity compared with the respective controls following exposure to serum starvation. Values are means ± S.E. of three independent experiments for each group. **p* < 0.05 versus the WT control; †*p* < 0.05 versus AKBI at 6 h.

signaling in AKBI MEFs. Importantly, these experiments also show that the absence of *IκBα* does not delay or alter the degree of NF-κB activation with an inflammatory stimulus.

Serum Starvation Results in Decreased Nuclear p65 in WT MEFs Prior to Cell Death—To determine whether the changes in NF-κB signaling occurred with exposure to serum starvation, the levels of nuclear p65 were evaluated at time points prior to initiation of apoptotic pathways. In WT MEFs, nuclear p65 decreased significantly after 30 min of serum starvation (Fig. 4, *A* and *B*) and remained diminished through 16 h of exposure (supplemental Fig. S4). A similar pattern was seen for both p50 and c-Rel (data not shown). In contrast, no significant change in the amount of nuclear p65 was seen in AKBI MEFs exposed to serum starvation (Fig. 4, *A* and *B*). To determine whether this lack of nuclear p65 is associated with changes in NF-κB activity, WT and AKBI MEFs were transfected with a NF-κB luciferase reporter and exposed to serum starvation. Following 6 h of serum starvation, NF-κB luciferase activity decreased by 30% compared with unexposed cells (*p* < 0.05), whereas no significant change was observed in AKBI MEFs (Fig. 4C). Overall, these findings suggest decreased NF-κB nuclear translocation and activation following exposure to serum starvation in WT cells but not in AKBI MEFs.

***IκBβ* Degradation Occurs following Oxidative Stress**—To evaluate the events upstream of changes in nuclear NF-κB subunits, the levels of cytosolic *IκBα* and *IκBβ* were assessed. No

significant change in cytosolic *IκBα* was found in WT cells exposed to serum starvation (Fig. 4, *D* and *E*, and supplemental Fig. S4). In contrast, a significant decrease in cytosolic *IκBβ* was seen in both WT and AKBI MEFs, although the decrease in AKBI MEFs was delayed, consistent with protein overexpression (Fig. 4, *D* and *F*). A similar pattern of *IκBβ* degradation was found in WT and AKBI MEFs exposed to glucose oxidase (Fig. 4, *G* and *H*). These findings suggest a delayed response to oxidative stress-induced NF-κB activation in cells that overexpress *IκBβ*.

***IκBβ* Overexpression and Absence of *IκBα* Prevent Serum Starvation Inhibition of NF-κB Activity**—To confirm that *IκBβ* overexpression is responsible for the resistance of AKBI MEFs to serum starvation-induced apoptosis, both WT and AKBI MEFs were subjected to *IκBβ* siRNA transfection. Knockdown of *IκBβ* in both WT and AKBI MEFs resulted in increased sensitivity to serum starvation-induced apoptosis in both cell lines (Fig. 5A). To further demonstrate the role of *IκBβ* in mediating the cellular response to oxidative stress, WT MEFs were transiently transfected with a plasmid containing either *IκBα* or *IκBβ*. Overexpression of either *IκBα* or *IκBβ* did not prevent caspase-3 cleavage (Fig. 5B). Therefore, WT MEFs were transiently transfected with a plasmid containing *IκBβ* and *IκBα* siRNA (Fig. 5C). Caspase-3 cleavage was attenuated in WT MEFs overexpressing *IκBβ* and *IκBα* silenced by siRNA (Fig. 5C). Thus, resistance to serum starvation-induced apoptosis is

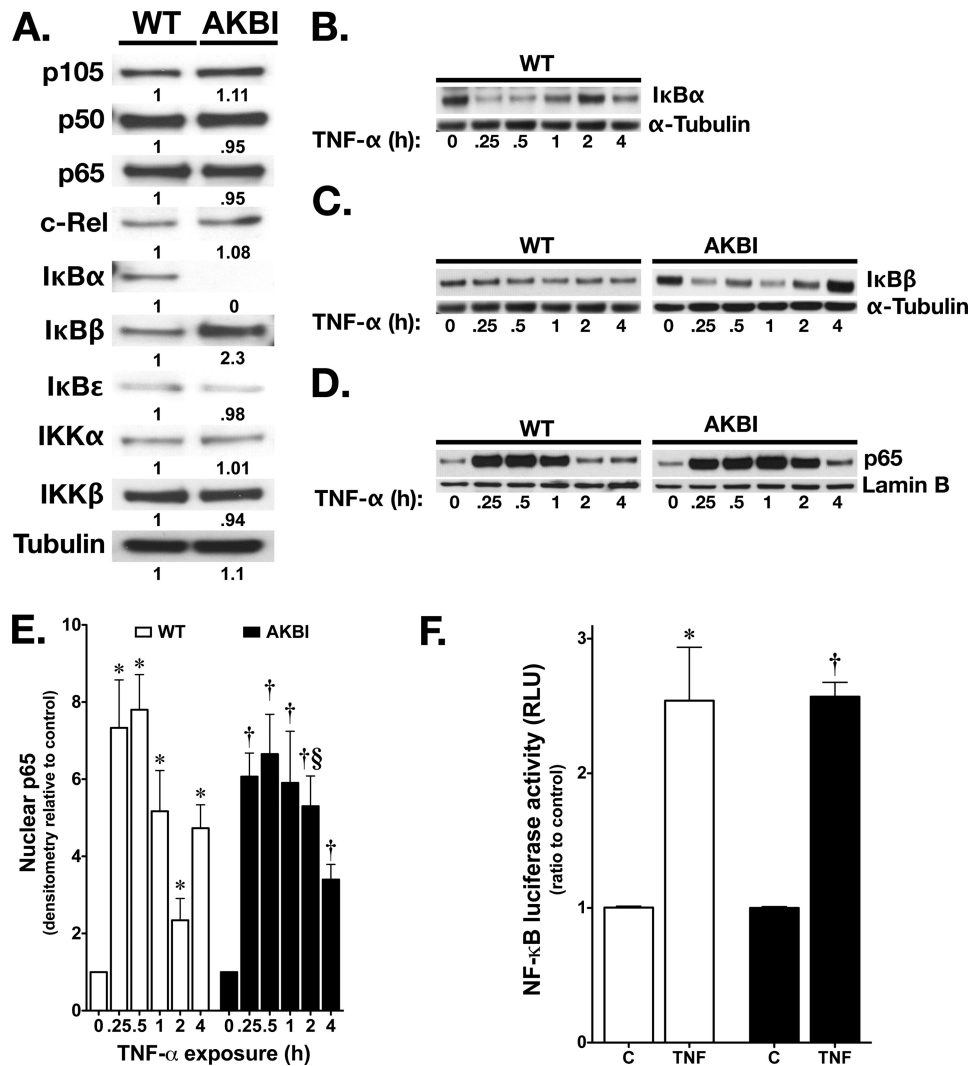


FIGURE 3. AKBI MEFs have intact canonical NF- κ B signaling. *A*, representative Western blot showing WT and AKBI lysate control levels of p105, p50, p65, c-Rel, I κ B α , I κ B β , I κ B ϵ , IKK α , and IKK β proteins with tubulin as a loading control. Densitometric evaluation of protein expression normalized to WT is provided. *B*, representative Western blot showing cytosolic I κ B α protein levels in WT MEFs exposed to TNF- α (10 ng/ml) with tubulin as a loading control. *C*, representative Western blot showing cytosolic I κ B β protein levels in WT and AKBI MEFs exposed to TNF- α (10 ng/ml) with tubulin as a loading control. *D*, representative Western blot showing nuclear p65 protein levels in WT and AKBI MEFs exposed to TNF- α (10 ng/ml) with lamin B as a loading control. *E*, densitometric evaluation of -fold increase in nuclear p65 in WT and AKBI MEFs exposed to TNF- α (10 ng/ml). Values are means \pm S.E. of three independent experiments for each group. *, $p < 0.05$ versus the WT control; \ddagger , $p < 0.05$ versus the AKBI control; \S , $p < 0.05$ versus WT at 2 h. *F*, WT and AKBI cells stably transfected with pNF- κ B-luc were exposed to TNF- α (10 ng/ml, 4 h). -Fold induction of photon emission was measured using a luminescence plate reader and normalized to *Renilla*. Values are means \pm S.E. of three independent experiments for each group. RLU, relative light units; C, control. *, $p < 0.05$ versus the WT control; \ddagger , $p < 0.05$ versus the AKBI control.

likely due to the combination of I κ B β overexpression and the absence of I κ B α in the AKBI MEFs. Finally, AKBI MEFs expressing I κ B α and transfected with I κ B β siRNA demonstrated increased caspase-3 cleavage after serum starvation (Fig. 5C). These data suggest that I κ B α cannot substitute for I κ B β in preventing serum starvation-induced apoptosis and that I κ B β overexpression alone is not sufficient to protect cells from serum starvation-induced cell death.

Decreases in Nuclear NF- κ B Results in Down-regulation of Specific Target Genes—To determine whether the decreased NF- κ B activity in WT MEFs exposed to serum starvation is associated with altered gene expression, a targeted gene array analysis was performed (mouse apoptosis RT2 profiler PCR array). In WT MEFs, serum starvation induced significant changes (defined as -fold change >2.5 , $p < 0.05$ versus the

unexposed control) in the expression of 12 of the 84 genes included in this array. Of these, 11 genes were up-regulated, whereas only one was down-regulated (Table 1). Importantly, all 11 of the up-regulated genes were pro-apoptotic, indicating activation of programmed cell death. In contrast, in AKBI MEFs, serum starvation induced significant changes in the expression of only five genes, with three genes up-regulated and two down-regulated (Table 2). These results show that WT MEFs are more sensitive to serum starvation-induced changes in gene transcription that are associated with changes in the NF- κ B signaling cascade. Importantly, these effects are attenuated in AKBI MEF cells. This clearly demonstrates the specificity of signaling associated with NF- κ B inhibitory proteins and shows that this specificity can alter apoptotic signaling and susceptibility to injury.

I κ B β Determines Survival after Oxidative Stress

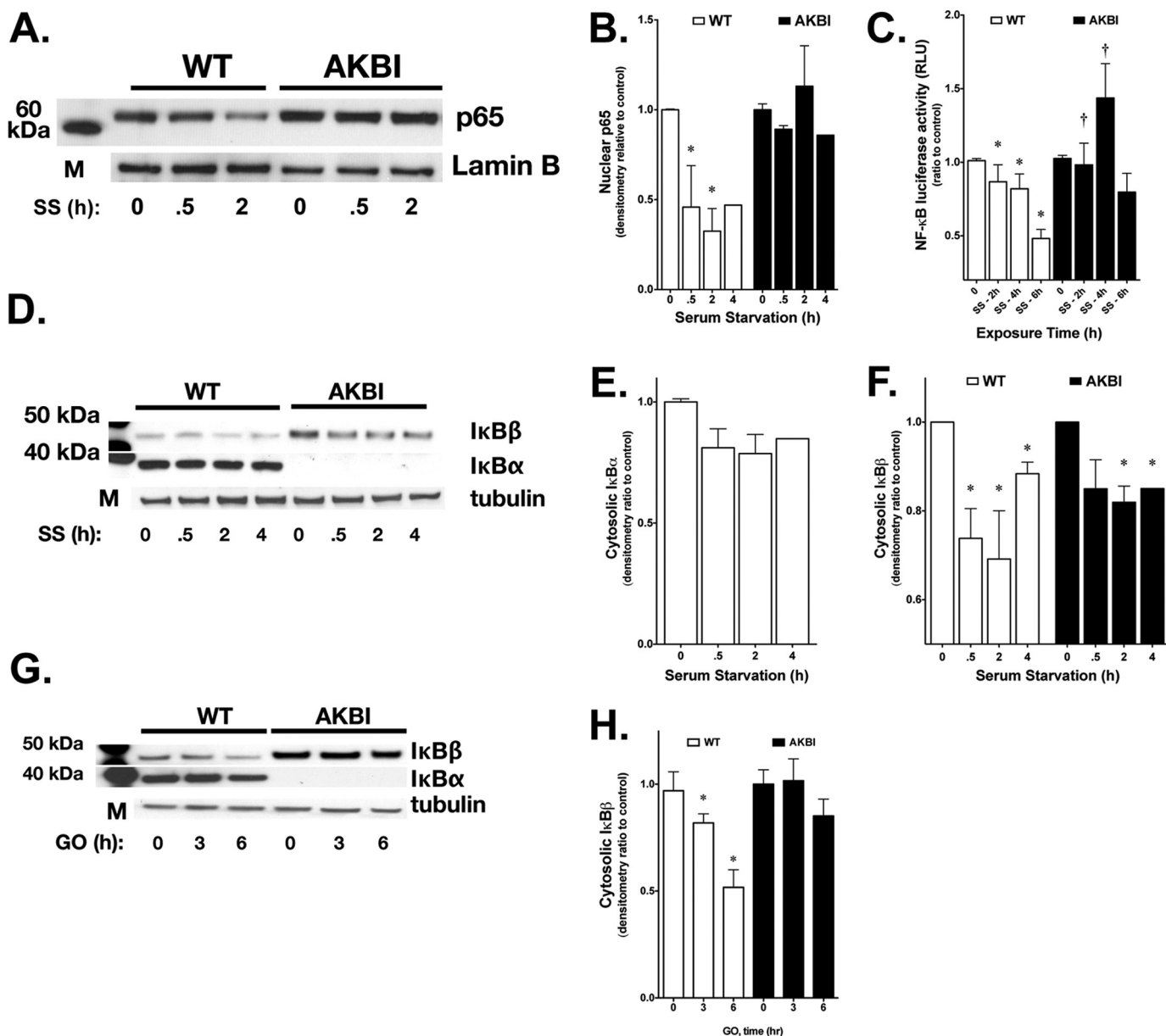


FIGURE 4. NF- κ B signaling differs between WT and AKBI MEFs exposed to oxidative stress. *A*, representative Western blot showing nuclear NF- κ B subunit p65 protein levels following exposure to serum starvation (SS) with lamin B as a loading control. *M*, marker. *B*, densitometric evaluation of -fold change in nuclear p65 after exposure to serum starvation. Values are means \pm S.E. of four independent experiments for each group. *, $p < 0.05$ versus the WT control. *C*, WT and AKBI cells stably transfected with pNF- κ B-luc and exposed to serum starvation. -Fold induction of photon emission was measured by an *in vivo* imaging system. Values are means \pm S.E. of three independent experiments for each group. RLU, relative light units. *, $p < 0.05$ versus the WT control; †, $p < 0.05$ versus the AKBI control. *D*, representative Western blot showing cytosolic I κ B α and I κ B β protein levels in WT and AKBI MEFs exposed to serum starvation with tubulin as a loading control. *E*, densitometric evaluation of -fold increase in cytosolic I κ B α after 0, 0.5, and 2 h of serum starvation. Values are means \pm S.E. of four independent experiments. *F*, densitometric evaluation of -fold change in cytosolic I κ B β in WT and AKBI MEFs after exposure to serum starvation. Values are means \pm S.E. of four independent experiments for each group. *, $p < 0.05$ versus the respective control. *G*, representative Western blot showing cytosolic I κ B α and I κ B β protein levels in WT and AKBI MEFs exposed to glucose oxidase (GO) with tubulin as a loading control. *H*, densitometric evaluation of -fold change in cytosolic I κ B β in WT and AKBI MEFs after exposure to glucose oxidase. Values are means \pm S.E. of four independent experiments for each group. *, $p < 0.05$ versus the WT control.

DISCUSSION

We have shown that NF- κ B activation, dictated by expression levels of I κ B family members, regulates cell survival in response to oxidative stress. The specificity of the NF- κ B response, as determined by I κ B family members, has been shown following inflammatory stress (18, 19). To our knowledge, this is the first documentation of I κ B proteins conferring specificity to the cellular response to oxidative stress.

Importantly, the NF- κ B signaling cascade converges on members of the I κ B family of inhibitory proteins. Functional differences between I κ B α and I κ B β exist, and the absence of either one drastically alters the cellular response to inflammatory stress (25). Recent investigations have utilized I κ B β ^{-/-} mice to dissect these functional differences. Interestingly, these mice demonstrate significantly enhanced resistance to endotoxic shock (18, 19). This resistance has been attributed to the

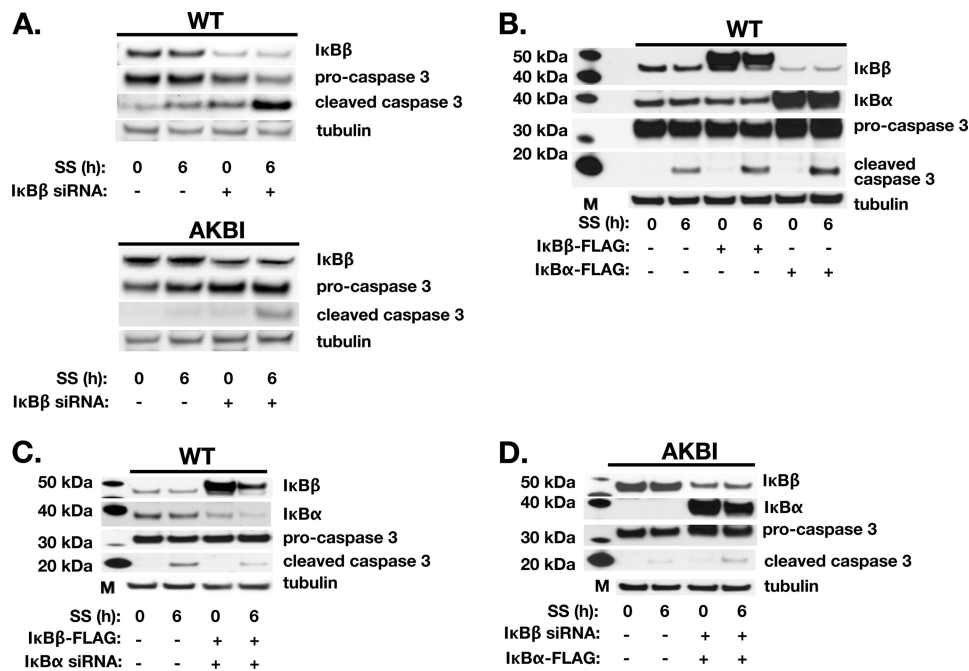


FIGURE 5. *IκBβ* overexpression combined with *IκBα* knockdown prevents serum starvation-induced apoptosis. *A*, representative Western blot showing levels of *IκBβ*, procaspase-3, and cleaved caspase-3 in WT and AKBI MEFs following transfection with *IκBβ* siRNA and subsequent exposure to serum starvation (SS) with tubulin as a loading control. *B*, representative Western blot showing levels of *IκBβ*, *IκBα*, procaspase-3, and cleaved caspase-3 in WT MEFs following transfection with an *IκBβ*-FLAG expression plasmid and *IκBα* plasmids and subsequent exposure to serum starvation with tubulin as a loading control. *M*, marker. *C*, representative Western blot showing levels of *IκBβ*, *IκBα*, procaspase-3, and cleaved caspase-3 in WT MEFs transfected with an *IκBβ*-FLAG expression plasmid and *IκBα* siRNA and subsequent exposure to serum starvation with tubulin as a loading control. *D*, representative Western blot showing levels of *IκBα*, *IκBβ*, procaspase-3, and cleaved caspase-3 in AKBI MEFs transfected with an *IκBα* expression plasmid and *IκBβ* siRNA and subsequent exposure to serum starvation with tubulin as a loading control.

TABLE 1

-Fold change in gene expression in WT MEFs exposed to serum starvation

Pro-apoptotic genes are indicated in boldface type. ↓, genes down-regulated by >2.5-fold versus the WT control; ↑, genes up-regulated by >2.5-fold versus the WT control.

Gene name (symbol)	WT serum starvation (-fold change)
Caspase-12 (<i>Casp12</i>)	6.6 ↑
Caspase recruitment domain family, member 9 (<i>Card6</i>)	5.5 ↑
TNF (ligand) superfamily, member 10 (<i>Tnfrsf10</i>)	3.7 ↑
Casp2 and RIPK1 domain-containing adaptor with death domain (<i>Cradd</i>)	3.7 ↑
Transformation-related protein 53-inducible nuclear protein 1 (<i>Trp53inp</i>)	3.6 ↑
Transformation-related protein 73 (<i>Trp73</i>)	3.0 ↑
Caspase-6 (<i>Casp6</i>)	2.9 ↑
Caspase-7 (<i>Casp7</i>)	2.9 ↑
Death-associated protein kinase 1 (<i>Dapk1</i>)	2.8 ↑
Caspase-9 (<i>Casp9</i>)	2.7 ↑
Fas (TNF receptor superfamily, member 6)	2.5 ↑
TNF receptor-associated factor 1 (<i>Traf1</i>)	3.1 ↓

TABLE 2

-Fold change in gene expression in AKBI MEFs exposed to serum starvation

Pro-apoptotic genes are indicated in boldface type. ↓, genes down-regulated by >2.5-fold versus the AKBI control; ↑, genes up-regulated by >2.5-fold versus the AKBI control.

Gene name (symbol)	AKBI serum starvation (-fold change)
Fas (TNF receptor superfamily, member 6)	2.9 ↑
Bcl-2/adenovirus E1B-interacting protein 3 (<i>Bnip3</i>)	2.7 ↑
Transformation-related protein 53-inducible nuclear protein 1 (<i>Trp53inp</i>)	2.7 ↑
TNF receptor superfamily, member 11b (<i>Tnfrsf11b</i>)	4.0 ↓
TNF receptor-associated factor 1 (<i>Traf1</i>)	4.2 ↓

differential expression of specific NF-κB target genes. The differential expression is explained by the unique affinity of *IκBα* and *IκBβ* for different NF-κB dimer combinations. Following nuclear translocation, these dimer combinations preferentially bind to unique consensus sequences.

Other investigators have indirectly implicated *IκBβ* in the cellular response to oxidative stress. Fan *et al.* (6) showed that

adult AKBI mice are more resistant to ischemia-reperfusion injury of the liver. This effect was attributed to the lack of *IκBα* expression in AKBI mice. Our results confirm that the absence of *IκBα* expression is central to the observed resistance to injury. In addition, our data suggest that *IκBβ* overexpression is necessary but not sufficient to prevent oxidative stress-induced apoptosis. In our study, WT MEFs overexpressing either *IκBα*

I κ B β Determines Survival after Oxidative Stress

or I κ B β remained sensitive to serum starvation, whereas silencing I κ B β alone increased this sensitivity (Fig. 5, A and B). Furthermore, WT MEFs were protected from serum starvation only in the setting of I κ B β overexpression combined with I κ B α knockdown (Fig. 5C). Likewise, AKBI MEFs became sensitive to serum starvation when I κ B β expression was silenced, an effect not rescued by I κ B α expression (Fig. 5D). Therefore, in this model, preventing oxidative stress-induced NF- κ B activity proceeding exclusively through I κ B β protects cells from apoptotic cell death.

These findings are similar to those of Biswas *et al.* (20, 26) in their study of mitochondrial stress-induced NF- κ B activity. Following mitochondrial stress, activated calcineurin dephosphorylates I κ B β , resulting in nuclear translocation of NF- κ B (26). Follow-up studies revealed a permissive role of I κ B β in signaling mitochondrial stress-induced NF- κ B activation (20). Silencing the “inhibitory” I κ B β prevented mitochondrial stress-induced NF- κ B activation rather than enhancing it. These findings, as well as our own, suggest a more complex role than previously appreciated for I κ B β in mediating NF- κ B activity. In the AKBI MEFs, overexpression of I κ B β prevented the loss of basal NF- κ B activity, suggesting a role of I κ B β in maintaining basal NF- κ B activity during oxidative stress. In our model, overexpression of I κ B β in WT MEFs was not sufficient to prevent loss of nuclear NF- κ B. However, when I κ B α was silenced, I κ B β overexpression prevented serum starvation-induced cell death. This suggests and points to a unique ability of I κ B β to promote survival through modulation of the expression of specific NF- κ B targets. More work is needed to determine whether this effect is due to the presence of nuclear I κ B β . Others have shown that I κ B β can exist in a complex with nuclear NF- κ B dimers bound to DNA (13–15). We speculate that I κ B β overexpression in the setting of oxidative stress maintains these complexes and subsequent gene expression. Modulating I κ B expression in the setting of oxidative stress represents a unique therapeutic target to prevent injury.

In a targeted gene array analysis, we found that the up-regulation of pro-apoptotic factors in WT MEFs was prevented in AKBI MEFs and that this correlated with increased apoptotic cell death in WT MEFs. Serum starvation is known to induce oxidative stress and activate the intrinsic pathway of apoptosis (21, 27, 28). As stated previously, a possible mechanism by which this occurs is through the ability of I κ B β to bind nuclear NF- κ B dimers on the promoters of specific target genes. The first indication that NF- κ B participates in cell survival was that RelA^{-/-} embryos demonstrated massive liver apoptosis (29). Further studies showed that NF- κ B regulated the expression of multiple anti-apoptotic factors, including *cIAP1*, *cIAP2*, *XIAP*, *cFLIP*, *Bcl-2*, *Bcl-x_L*, *TRAF1*, and *TRAF2* (30, 31). More work is needed to identify whether I κ B β specifically regulates the expression of these genes during oxidative stress.

In summary, we have shown a marked resistance to serum starvation-induced apoptotic cell death in MEFs that overexpress I κ B β in the absence of I κ B α . This finding was associated with maintenance of basal NF- κ B nuclear localization and activity and lack of up-regulation of pro-apoptotic genes in AKBI MEFs. In oxidative stress, preventing the loss of base-line

NF- κ B activity through enhancing I κ B β expression may serve as a therapeutic target to enhance cell survival.

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