Cell-based NF-κB/I-κBα signaling

NF-κB/I-κBα signaling in HeLa cells: Phosphorylation and degradation

Introduction

Inactive NF-κB is located in the cytosol in a complex with the inhibitory protein I-κBα. A variety of extracellular stress-related signals, including inflammatory cytokines, free radicals, UV irradiation, and bacterial or viral antigens can initiate phosphorylation and activation of IκB kinase (IKK). Activated IKK phosphorylates I-κBα, which subsequently dissociates from NF-κB. Phosphorylated IκBα is ubiquitated and degraded by the proteosome. Phosphorylated NF-κB is then translocated into the nucleus where it acts as a transcriptional activator.

The NF-κB pathway plays an important role in regulating immune responses to infection, and deregulation of NF-κB has been linked to several diseases, including cancer, and autoimmune diseases.

Materials used

HeLa cells are available from ATCC. Cell culture media and additives are available from Invitrogen. TNFα and TPCA-1 are available from Sigma. AlphaScreen SureFire phospho NF-κB, total NF-κB, phospho I-κBα, and total I-κBα assay kits are available from PerkinElmer. Western blotting detection reagents and secondary antibody-HRP conjugates are available from Perkin Elmer. Tissue culture plasticware is from Nunc, and AlphaScreen SureFire assay plates are available from PerkinElmer.

Results

Stimulation time point selection

HeLa cells were stimulated with 10 ng/mL TNFα for various lengths of time at room temperature. The cells were lysed, and the lysates were analysed for phosphorylated and total levels of both NF-κB (Figure 1) and I-κBα (Figure 2), in order to determine the optimal time point for measuring phosphorylation of both NF-κB and I-κBα.

Under the conditions tested, maximal phosphorylation of NF-κB was observed after 5 minutes of stimulation. Elevated phosphorylation levels were observed until 10 minutes after which levels decreased. Total protein levels of NF-κB remained unchanged for the duration of the time course stimulation.

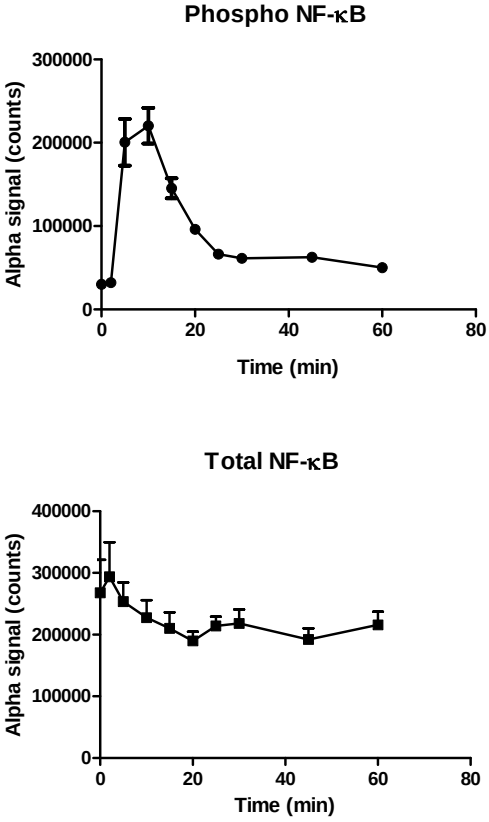

Figure 1

Similarly, I-κBα phosphorylation stimulated by TNFα was also transient. Maximal levels of phosphorylation were observed at 5 minutes, and returned to basal levels after 15 minutes. Furthermore, marked differences in the level of total I-κBα were observed. Levels of I-κBα rapidly decreased after 5 minutes of TNFα stimulation, and then gradually started to increase again after 30 min. I-κBα phosphorylation and degradation were confirmed by western blotting (Figure 2). To test if

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the decrease in total I-κBα observed was a result of ubiquitination blocking the antibody recognition, a different antibody - raised to a distal epitope – was also tested in a Western blot. A similar depletion of total I-κBα result was observed (data not shown).

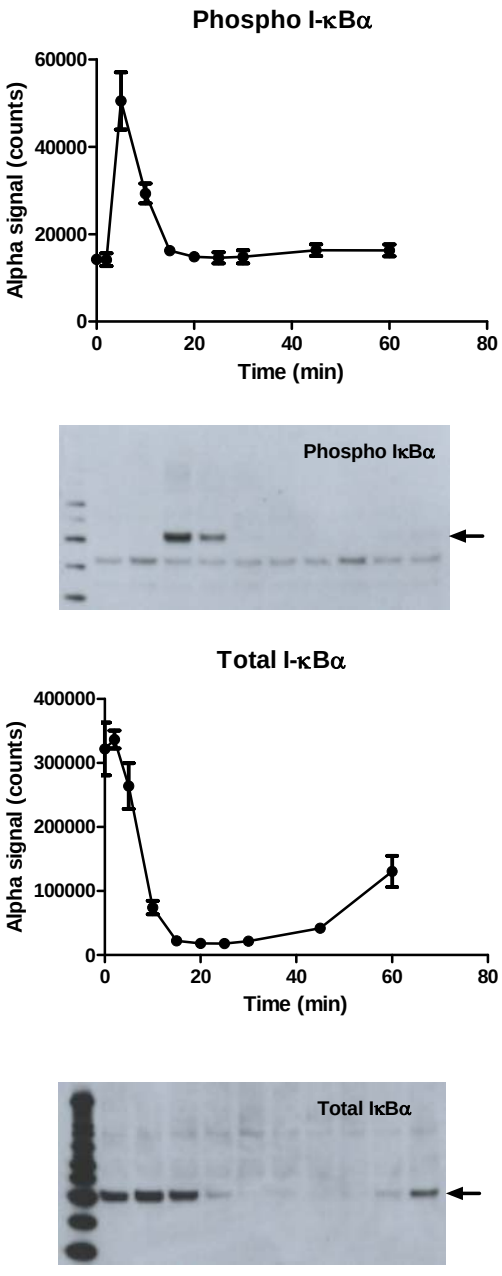

Figure 2

Based on these results, 5 minute stimulation with TNFα at room temperature was selected as a useful time point for further analysis of NF-κB and I-κBα phosphorylation.

Dose-dependent stimulation with TNFα

HeLa cells were stimulated for 5 minutes with various concentrations of TNFα, then lysed. The lysates were analysed for phosphorylated and total levels of both NF-κB and I-κBα. TNFα-induced phosphorylation of both proteins in a dose dependent manner with EC_{50} values of 0.74 ng/mL and 2.4ng/mL for NF-κB and I-κBα, respectively Figure 3).

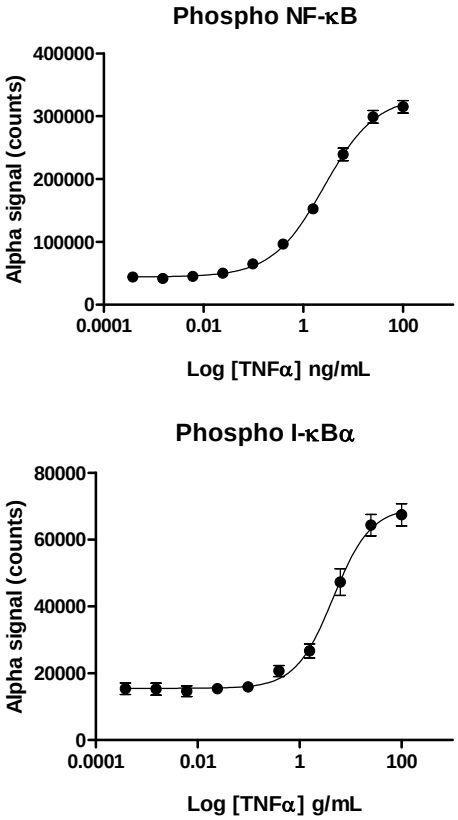

Figure 3

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Total levels of NF-κB protein remained unchanged as expected, at all concentrations of TNFα treatment. However, total levels of I-κBα protein were slightly reduced at higher concentrations of TNFα, suggesting that at higher concentrations of TNFα, I-κBα protein degradation may occur at a timepoint as early as 5 minutes after stimulation (Figure 4).

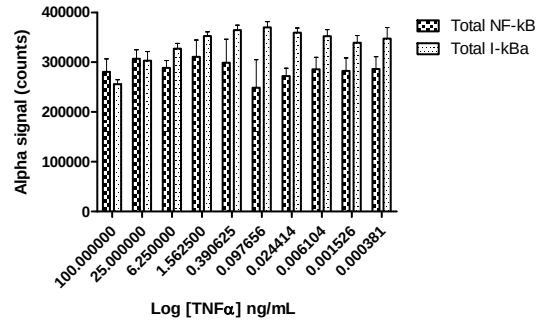

Figure 4

Inhibition with TPCA-1

The effect of the I-κB kinase 2 (IKKB) selective inhbitor, TPCA-1, on NF-κB and I-κBα phosphorylation was examined. HeLa cells were treated with various concentrations of TPCA-1 for 60 minutes, and then stimulated with 10 ng/mL TNFα for 5 minutes. The cells were lysed, and the lysates were analysed for modulation of NF-κB and I-κBα (Figure 5). TPCA-1 was found to inhibit phosphorylation of both targets in a dose dependent manner with IC_{50} values of 5.8 µM and 0.67 µM for NF-κB and I-κBα, respectively.

Again, total levels of NF-κB protein were unchanged. Total levels of I-κBα protein were slightly reduced at lower concentrations of inhibitor (data not shown).

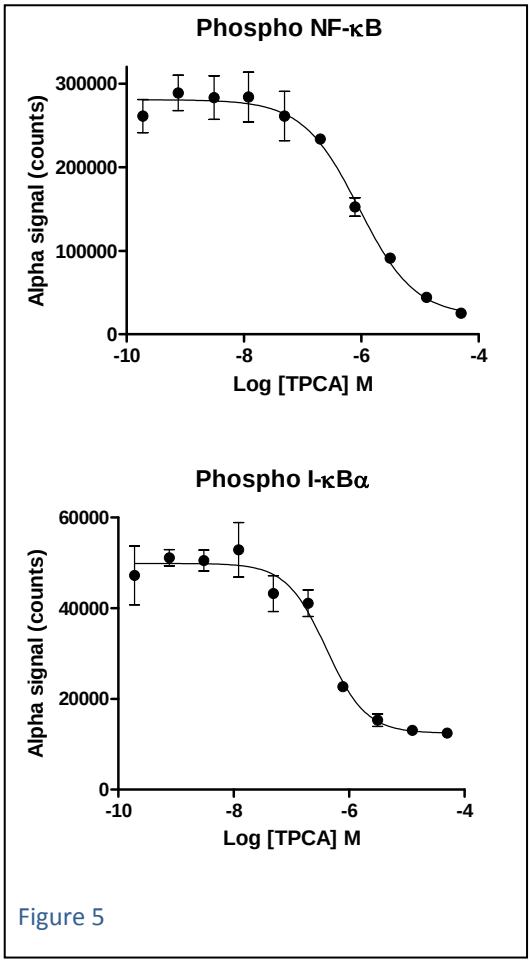

Figure 5

IκBα protein degradation

In the previous experiments, 5 min stimulation with TNFα was used, as this time point resulted in maximal phosphorylation of both proteins. As the NF-κB/I-κBα pathway is regulated by degradation of I-κBα as well as phosphorylation events, the dose response experiments were repeated, stimulating for 15 minutes with TNFα rather than 5 minutes, to ensure maximal loss of I-κBα signal. TNFα treatment for this period of time induced dose-dependant I-κBα degradation (EC_{50} 0.34 ng/mL), doses comparable to the doses observed for NF-κB phosphorylation (Figure 6). TNFα induced I-κBα protein degradation could be inhibited by pre-treatment with TPCA-1, with an IC_{50} of 1.3 µM, a value similar to that observed from direct

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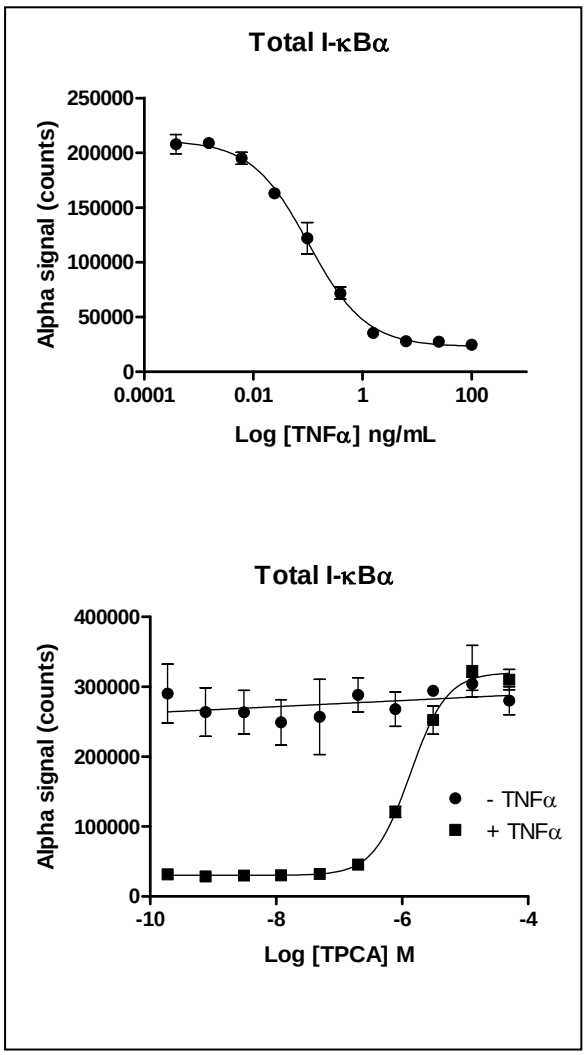
inhibition of phosphorylation of I-κBα measured at 5 min (Figure 5).



Conclusions

Modulation of the NF-κB/I-κBα pathway is readily detectable in HeLa cells in response to TNFα treatment. Using AlphaScreen SureFire assays, phosphorylated NF-kB and I-κBα can be detected. For I-κBα, both phosphorylation and also total protein levels could be used as a marker for pathway regulation. In contrast, NF-kB levels were not modulated by TNFα, and phosphorylation was the most useful marker.

Ordering Information

All cell lines are available from ATCC (www.atcc.org).

AlphaScreen® SureFire® cellular assay kits, available in 500pt, 10,000pt and 50,000pt pack sizes, are from PerkinElmer.

Catalog#

Note: These kits must be used in conjunction with the AlphaScreen protein A

TGR TECHNICAL NOTE