Rapid Up-regulation of IκBβ and Abrogation of NF-κB Activity in Peritoneal Macrophages Stimulated with Lipopolysaccharide*

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Marta Velasco†, María J. M. Díaz-Guerra‡, Paloma Martín-Sanz, Alberto Álvarez†, and Lisardo Boscá‡

From the Instituto de Bioquímica (Consejo Superior de Investigaciones Científicas) and ‡Centro de Citometría de Flujo, Facultad de Farmacia, 28040 Madrid, Spain

Lipopolysaccharide (LPS) administration to mice elicited the activation of nuclear factor κB (NF-κB) in several tissues including liver and macrophages. Maximal activation was observed 1 h after treatment but declined at 3 and 6 h. The levels of IκBα and IκBβ were analyzed during this period in an attempt to correlate NF-κB activity with IκB resynthesis. Degradation of IκBα was very rapid and was followed by recovery 1 h after LPS administration. IκBβ degradation, which has been associated with persistent NF-κB activation, was complete at 1 h. However, a rapid recovery of IκBβ in these tissues was observed at 3 h in parallel with the abrogation of NF-κB activity. Immunolocalization of newly synthesized IκBβ by confocal microscopy revealed its preferential accumulation in the cytosol. Analysis of IκBβ by Western blot using high resolution polyacrylamide gel electrophoresis showed the presence of two bands in cytosolic extracts of LPS-treated macrophages at 3 h, but only one band with the same mobility as the control was detected at 6 h. Moreover, treatment of extracts of resynthesized IκBβ with alkaline phosphatase resulted in the accumulation of the protein of slightly higher electrophoretic mobility, indicating the prevalence of a rapid phosphorylation of the newly synthesized IκBβ. At the mRNA level, up-regulation of IκBβ was observed in macrophages stimulated for 1 h with LPS. When the effect of pro-inflammatory cytokines was investigated, tumor necrosis factor α, but not interleukin-1 or interferon-γ, promoted an important degradation of IκBβ followed by an increase in the mRNA at 1 h. These results suggest the existence of LPS- and tumor necrosis factor α-specific pathways involved in a rapid IκBβ degradation and resynthesis and might explain the transient period of activation of NF-κB in these tissues upon stimulation with these factors. This rapid control of NF-κB function may contribute to the attenuation of the inflammatory response of these cells.

Nuclear factor κB (NF-κB)3 participates in the regulation of the expression of multiple immediate early genes involved in the immune, acute phase, and inflammatory responses (1). NF-κB is a heterodimer of proteins of the Rel family of transcription factors. In mammalian cells, they include p65 (Rel A), Rel B, the proto-oncogene c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) (1, 2). NF-κB proteins are constitutively present in the cell, but they are retained in the cytoplasm associated with inhibitory proteins known as IκB (3, 4). Activated NF-κB complexes, typically composed of p50 and p65, are translocated to the nucleus in response to mitogens, cytokines (IL-1β, IL-2, and TNF-α), and bacterial lipopolysaccharide and lipopeptides (1, 5–8). Activation of NF-κB appears to require phosphorylation and degradation of the IκB proteins, thereby allowing the rapid translocation of NF-κB from the cytoplasm to the nucleus (7, 9–11).

Several IκB proteins have been characterized including IκBα, IκBβ, IκBγ, and the candidate oncogene Bcl-3 (3, 4, 12). All these proteins share a characteristic ankyrin repeat motif, which is required for the interaction with Rel proteins, and a C-terminal PEST sequence presumably involved in protein targeting and degradation (13). Different kinases have been involved in IκBα phosphorylation, but the observation that antioxidants and alkylating agents inhibit the phosphorylation and subsequent degradation points to a common unidentified IκBα kinase (14, 15). Phosphorylation of specific residues seems to be the signal for ubiquitin conjugation followed by degradation via the 26 S proteasome (16, 17). Degradation of IκBα is rapidly followed by induction of IκBα mRNA through a mechanism dependent on the binding of NF-κB to the κB sequences present in the promoter of the IκBα gene (18, 19). This newly synthesized IκBα resets the NF-κB switch in the cytoplasm and possibly in the nucleus (20), although in some cases IκBα resynthesis is not sufficient to suppress nuclear NF-κB activity (12, 21). IκBβ has been cloned recently (12) and, together with IκBα, is the main regulator of NF-κB activity through the interaction with the same Rel proteins (2). It has been proposed that IκBβ degradation causes a sustained activation of NF-κB due to the large lag period of IκBβ resynthesis (12, 21, 22). To determine whether these observations are specific of some cell types or represent a general mechanism of NF-κB activation, we investigated IκBα and IκBβ turnover in an experimental model of murine septic shock and in cultured peritoneal macrophages triggered with different stimuli. Our results show a rapid IκBβ degradation followed by a fast recovery, both in liver and in macrophages. This recovery of IκBβ contrasts with the results obtained in LPS-stimulated lymphoid cells, where absence of IκBβ was observed for large periods of time (12). In our experimental model, an increase of IκBβ mRNA was detected as early as 1 h after stimulation and paralleled the resynthesis of IκBβ levels and the fall in NF-κB activity.

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† Both authors contributed equally to this work.
‡ To whom correspondence should be addressed: Instituto de Bioquímica, Facultad de Farmacia, 28040 Madrid, Spain. Fax: 34-394-1782; E-mail: boscal@eucmax.sim.ucm.es.

1 The abbreviations used are: NF-κB, nuclear factor κB; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; INOS, type II NO synthase; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; Abs, antibodies; IFN, interferon; TPCK, tosylphenylalanyl chloromethyl ketone.

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MATERIALS AND METHODS

Chemicals—Cytokines were from Boehringer Mannheim. Polymerase chain reaction reagents were from Perkin-Elmer. Lipopolysaccharide (LPS) from Salmonella typhimurium and other reagents were from Sigma. Cell culture reagents were from BioWhittaker, Inc. (Walkersville, MD). Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animal Treatment—Septic shock was induced in mice after intra-peritoneal injection of 0.5 ml of a solution containing LPS (1 mg/kg of body weight) in saline. Animals were anesthetized with ether and immediately sacrificed. Tissues were processed immediately after extraction.

Preparation of Macrophages—Elicited peritoneal macrophages were prepared from male mice 4 days after intra-peritoneal inoculation of 1 ml of 10% thioglycollate broth. Cells were seeded at 1.5 × 10^5 in 6-cm plates and cultured with RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics at 37 °C in an atmosphere of humidified 5% CO2. After incubation for 1 h, nonadherent cells were removed, and remanent cells were cultured and stimulated for different periods of time in phenol red-free RPMI 1640 medium lacking serum.

RNA Extraction and Northern Blot Analysis—Total RNA was extracted from 1.5 × 10^5 cells or from 50 mg of tissues following the guanidinium thiocyanate method (23). After electrophoresis in a 0.9% agarose gel containing 2% formaldehyde, the RNA was transferred to a Nytran membrane (NY 13-N; Schleicher & Schuell, Inc., FRG) with agarose gel containing 2% formaldehyde. The films was performed by laser densitometry (Molecular Dynamics, Sunnyvale, CA) using the hybridization with a ribosomal 18 S probe as internal standard. Various exposition times of the micrograph films were used to ensure that bands were not saturated. Results are expressed as arbitrary units as the ratio of IκB/ribosomal 18 S RNA level.

Preparation of Cytosolic and Nuclear Extracts—A modified procedure developed on the basis of Schreiber et al. was used. Cells were stimulated for the indicated period of time and the cell layers were washed with ice-cold PBS and fixed for 2 min with methanol (−20°C). Fixed cells were blocked for 1 h at 4°C with 10% fetal calf serum in PBS. The cell layers were stimulated for the indicated period of time, and the cell layers were washed with ice-cold PBS and fixed for 2 min with methanol (−20°C). Fixed cells were blocked for 1 h at 4°C with 10% fetal calf serum in PBS. The cell layers were washed with ice-cold PBS and analyzed in a MRC-100 confocal microscope (Bio-Rad).

RESULTS

IκB is Up-regulated after LPS Treatment in Vivo and ex vivo—It has been suggested that the persistent NF-κB activation observed in B lymphocytes in response to LPS is due to a selective degradation and delayed resynthesis of IκBα (12). Indeed, administration of LPS to mice induces NF-κB activation in several tissues, including resident peritoneal macrophages and liver (5, 26, 27). Using this animal model of septic shock, we analyzed the process of NF-κB activation together with IκB degradation and synthesis. As Fig. 1A shows, a peak of NF-κB binding was observed 1 h after LPS administration both in peritoneal macrophages and in liver. This response decreased at 3 and 6 h and was completely absent in samples obtained at 18 h. Because NF-κB activation is largely depended on IκB degradation, we analyzed the levels of IκBα and IκBβ at several sampling times. In liver, IκBα was lost at 20 min and rapidly recovered at 5 h (Fig. 1B). Interestingly, IκBα degradation and resynthesis was delayed with respect to IκBα, recovering basal levels at 6 h. However, the recovery of IκBα and IκBβ in peritoneal macrophages from these animals was more rapid (Fig. 1B), suggesting a differential kinetic control of this process among cells.

To assess whether this extremely rapid IκBα up-regulation was a peculiarity of the in vivo experimental model of septic shock, cultured peritoneal macrophages were stimulated with LPS, and NF-κB activity and IκB levels were determined. As
Up-regulation of IκBβ after LPS and TNF-α Triggering

Proteasome inhibitors block IκBβ degradation in cultured peritoneal macrophages stimulated with LPS

| Treatment        | IκBβ level |
|------------------|------------|
|                  | 20 min | 1 h | 4 h |
| None             | 100    | 101 | 101 |
| Calpain I inhibitor, 40 μM | 105   | 107 | 110 |
| TPCK, 40 μM     | 109    | 109 | 112 |
| LPS, 1 μg/ml    | 18     | <1  | 71  |
| Calpain I inhibitor, 40 μM | 91    | 89  | 104 |
| TPCK, 40 μM     | 94     | 92  | 99  |

Fig. 1. NF-κB, IκBα, and IκBβ levels in peritoneal macrophages and liver of mice under septic shock conditions. Thioglycollate-elicited mice were intraperitoneal-injected with LPS (1 mg/kg), and samples of liver and peritoneal macrophages were collected at the indicated times. NF-κB activity was measured in nuclear extracts (3 μg of protein) after binding to the NF-κB motif of the iNOS promoter. Panel A, upper and lower arrows indicate the specific binding complexes. Panel B, the amount of IκBα and IκBβ was determined by Western blot in cytosolic extracts (15 μg of protein) corresponding to the same samples analyzed for NF-κB binding. Results show a representative experiment out of three.

Fig. 2. IκBβ resynthesis in cultured peritoneal macrophages challenged with LPS. Peritoneal macrophages were kept in culture (1.5 x 10⁶ cells), and after stimulation with 1 μg/ml of LPS for the indicated period of time the cells were homogenized. Panel A, nuclear extracts were used to determine NF-κB binding by EMSA, and the amount of IκBβ was evaluated by Western blot in the corresponding cytosolic extracts (15 μg of protein). Panel B, nuclear extracts from cells treated for 1 h with LPS were pooled and used to identify by supershift the proteins present in the bands. Panel C, cytosolic extracts from control or LPS-treated cells (panel A, 3 and 6 h) were incubated with heat-inactivated (AP−) or active (AP+) agarose-immobilized alkaline phosphatase and size separated in a high resolution SDS-polyacrylamide gel electrophoresis. Arrows indicate the IκBβ-immunodetected bands. Results show a representative experiment out of four.

Fig. 3. Immunolocalization of IκBα and IκBβ by confocal microscopy. Cultured peritoneal macrophages were stimulated for the indicated period of time with 1 μg/ml of LPS. After fixing and permeabilization, the cells were incubated with anti-IκBα or anti-IκBβ Ab. Visualization of the proteins was carried out using a fluorescein-labeled secondary Ab. Bars correspond to 50 μm.

TABLE I

| Treatment        | IκBβ level |
|------------------|------------|
|                  | 20 min | 1 h | 4 h |
| None             | 100    | 101 | 101 |
| Calpain I inhibitor, 40 μM | 105   | 107 | 110 |
| TPCK, 40 μM     | 109    | 109 | 112 |
| LPS, 1 μg/ml    | 18     | <1  | 71  |
| Calpain I inhibitor, 40 μM | 91    | 89  | 104 |
| TPCK, 40 μM     | 94     | 92  | 99  |

Increased mobility of the protein after treatment with immobilized alkaline phosphatase (Fig. 2C). Interestingly, the main IκBβ species detected in control cells corresponded to the phosphorylated state of the protein as deduced by the shift after phosphatase treatment. Quantitation of the relative intensity of the two bands observed in samples from cells treated for 3 h with LPS showed a 78 and 22% distribution for the upper and lower bands, respectively. However, in samples analyzed after 6 h of LPS treatment, the upper band systematically represented >90% of the distribution, indicating the prevalence of the phosphorylated form in the cytosol. The presence of non-phosphorylated IκBβ species has been related to the formation of IκBβ-protected NF-κB active complexes both in the cytosol and in the nucleus (22). Therefore, to investigate whether these protected ternary complexes could be present in the nuclei of LPS-activated cells, a supershift EMSA was performed with anti-IκBβ Ab after incubation of the nuclear extracts from cells treated for 1 or 3 h with LPS. However, the electrophoretic profile of NF-κB binding was not affected. Moreover, the minimal amount of IκBβ detected in the nuclear extracts corresponded to the phosphorylated form. These results indicate that the level of IκBβ present in the nucleus was very low, always corresponding to the active form of the protein (not shown, see next section).

To determine the contribution of the proteasome to IκBβ degradation, experiments were done in the presence of several proteasome inhibitors, and the amount of IκBβ was determined.
by Western blot. As Table I shows, these inhibitors prevented IκBβ degradation at the time that prevented NF-κB activation (not shown).

IκBβ Accumulates in the Cytosol of LPS-treated Macrophages—The degradation and resynthesis of IκBα and IκBβ in cells treated with LPS was also investigated in situ using fluorescence confocal microscopy. As Fig. 3 shows, IκBα and IκBβ were undetectable at 30 min. Newly synthesized IκBα was detected at 1 h after LPS treatment, and at 4 h the protein was present both in the cytosol and in the nucleus. A quantitative analysis of the subcellular distribution of the fluorescence is shown in Fig. 4. When the immunofluorescence associated with IκBβ was analyzed (Fig. 3), a complete absence of staining was observed in cells treated for 1 h with LPS, followed by a resynthesis of the protein that accumulates in the cytosol. These time courses of resynthesis were in agreement with the immunodetection of the protein by Western blot analysis. Whereas IκBα was observed both in the cytosol and nucleus, IκBβ was detected in the cytosol in agreement with the rapid phosphorylation of this protein, which blocks the nuclear localization signal domain of the NF-κB-Rel complexes. The quantitative analysis of these data is reported in Fig. 4.

IκBα mRNA Levels Are Increased by LPS—The steady-state levels of IκBα and IκBβ mRNA were determined in order to better assess the recovery of the corresponding proteins. As Fig. 5, A and B, show and in agreement with previous reports, IκBα mRNA was rapidly up-regulated after treatment of cultured macrophages with LPS (12, 27). Interestingly, IκBβ mRNA also increased in response to LPS, although the changes were lower and delayed with respect to the IκBα levels (peak values were obtained at 1 and 4 h for IκBα and IκBβ, respectively). To confirm the specificity of the mRNA detected by the probes, total RNA from selected murine tissues was examined by Northern blot, and cross-hybridization with both probes was accomplished. As Fig. 5C shows, IκBα and IκBβ mRNAs were expressed at different levels in several tissues in agreement with a previous report (12). IκBα was very abundant in spleen, whereas IκBβ exhibited a high expression in testis. However, both IκBα mRNA increased in the liver of animals after LPS treatment for 4 h (Fig. 5C), supporting the results shown in Fig. 1 at the protein level.

TNF-α but Not IL-1β or IFN-γ Induces IκBβ Degradation and IκBβ mRNA Up-regulation in Macrophages—NF-κB activation requires phosphorylation, targeting, and degradation of the IκB components of the heteromeric complexes. Therefore, the measurement of the IκBα and IκBβ mRNA and protein levels provides useful criteria for the assessment of their rate of resynthesis and the turn-off of the NF-κB activation process. To investigate the effect of pro-inflammatory cytokines on the levels of IκBβ, macrophages were stimulated with TNF-α, IFN-γ, IL-1β, or a combination of them, and the amount of IκBβ was quantified by Western blot. As Fig. 6 shows, only cells treated with TNF-α exhibited a decrease in IκBβ levels (46% of the control value) 1 h after stimulation, whereas recovery was observed at 4 h. Analysis of IκBβ on a high resolution gel showed the presence of 16% nonphosphorylated protein at 4 h (not shown). Simultaneous triggering with TNF-α, IL-1β, and

**Fig. 4.** *Quantitative analysis of IκBα and IκBβ levels in intact cells.* The fluorescence intensity of cells treated as indicated in the Fig. 3 legend was digitalized using the software of the confocal microscope. The fluorescence values associated to the cytosol and nucleus were determined. Results show the average fluorescence (± S.E.) of at least 12 cells for each condition. * and ** denote p < 0.05 and p < 0.01, respectively, corresponding to values at zero time. a.u., absorbance units.

**Fig. 5.** IκBα and IκBβ mRNA are up-regulated in cultured peritoneal macrophages challenged with LPS. Panels A and B, cultured peritoneal macrophages were stimulated with 1 μg/ml LPS, and the mRNA levels corresponding to IκBα (open bars) and IκBβ (solid bars) were determined by Northern blot after normalization for the content of ribosomal 18 S RNA. Results were expressed as the mean ± S.E. of three experiments, and the values were referred to the time 0 h condition (panel A). The specificity of the IκBα and IκBβ mRNA detected was determined by cross-hybridization of a membrane containing 20 μg of total RNA of the indicated tissues with each probe. Results were not affected by the order of the sequential hybridization. When the effect of LPS on liver IκB mRNA levels was measured, this was intraperitoneal-injected at 1 mg/kg, and liver samples were collected after 4 h (panel C). Results show a representative experiment out of three. * denotes p < 0.005 with respect to the corresponding values at zero time.
IFN-γ, a condition that appears to potentiate the expression of some genes dependent on NF-κB activation, did not modify the response to TNF-α alone. The mRNA levels of IkBa and IkBβ were measured at 1 and 4 h (Fig. 7). In agreement with the effects of TNF-α at the protein level, an increase of IkBβ mRNA at 1 h (3-fold) and at 4 h (5.4-fold) was observed. Interestingly, challenge of macrophages with TNF-α, IFN-γ, and IL-1β resulted in a synergistic effect on IkBa mRNA up-regulation but not on IkBβ, suggesting a different transcriptional control of both genes.

**DISCUSSION**

Activation of NF-κB constitutes an important step in the course of several immune and inflammatory responses, including septic shock (1, 2, 8, 11). Two main regulatory mechanisms of NF-κB activity have been recognized. One is the precise nucleotide sequence of the κB motif to which NF-κB binds, the important differences existing in the transcriptional activity depending on variations in the consensus sites and in the flanking regions (28, 29). The other involves the association of NF-κB with inhibitory subunits such as the various forms of IkB proteins and the formation of an inactive complex in the cytosol (3, 11). Specific interactions between IkB and NF-κB proteins have been described. For example, IkBa and IkBβ strongly bind to p65 and c-Rel but not to the p50 component of the complex (3, 4). In addition to this, a cell-specific pattern of expression of members of the IkB family has been observed (12), and therefore, NF-κB activity depends ultimately on the balance between the rates of degradation and resynthesis of each IkB protein. We have used the κB sequence corresponding to the murine iNOS promoter, a gene for which transcription requires NF-κB activation and that exhibits a transient expression in the tissues examined (26, 27). Our data show a complete degradation of IkBβ in liver and in peritoneal macrophages of animals challenged with LPS as well as in cultured macrophages. This degradation was very rapid since a complete loss of immunodetected protein in the Western blot was observed less than 1 h after stimulation. The best known pathway of IkB degradation is that of IkBa. Phosphorylation of IkBa is a necessary requisite for its proteolytic degradation that is essential for in vitro NF-κB activation (10). The identification of the enzymes involved in IkBa phosphorylation points to several protein kinases, including casein kinase II and mitogen-activated protein kinase (10, 29–31). Interestingly, these data suggest that IkBa might be targeted by several protein kinases depending on the extracellular stimuli. However, the mechanisms that control IkBβ targeting and degradation still remain unidentified, although the proteasome is required for this process (Ref. 12 and this data).

Resynthesis of IkBa is directed by NF-κB activation (18). However, the regulation of the transcriptional activity of the IkBβ promoter is poorly characterized, and only indirect data are available. In B cell lines, LPS and IL-1 promoted a persistent IkBβ degradation compatible with a sustained NF-κB activation for at least 48 h, whereas phorbol esters and cytokines such as TNF-α did not affect IkBβ levels and produced only a transient activation of NF-κB (12). Also, activation of human vascular endothelial cells with phorbol esters and TNF-α produced a persistent activation of NF-κB (more than 20 h) that paralleled IkBβ degradation, whereas IL-1 produced only a transient activation associated with a rapid recovery of IkBβ (21); moreover, the co-stimulatory signal elicited by CD28 engagement in T cells produced a rapid and persistent degradation of IkBβ that contributed to the activation of several NF-κB/Rel heterodimers (32). Opposite of these cases, our data clearly show that in peritoneal macrophages treated with LPS or TNF-α or in liver from animals suffering septic shock, a rapid resynthesis of IkBβ occurred concomitantly with the decrease of NF-κB activity. However, a residual NF-κB activity still persists in macrophages after 6 h of treatment, suggesting...
that the levels of IκB are not sufficient to dissociate NF-κB from the DNA either because the turnover of IκB in the cytosol is rapid or because of an improved stability of the NF-κB-DNA complexes present in the nucleus of these cells. At the mRNA level, up-regulation of IκBα was observed at 1 h after stimulation, immediately following IκBα induction. Taken together, these data suggest the existence of pathways (dependent on LPS and TNF-α in our models) that can rapidly regulate IκBα transcription and in this way contribute to attenuate NF-κB-dependent responses. Moreover, a certain cell specificity exists in the control of IκBα degradation in view of the opposite results observed in preB cells (70Z/3 cells) and in endothelial cells upon challenge with the same array of pro-inflammatory cytokines and phorbol esters (12, 21).

The rapid re-synthesis of IκBα observed in our experimental model parallels the fall in NF-κB activity. However, recent results indicate that the phosphorylation state of the newly synthesized IκBα protein might influence its interaction with NF-κB (22, 33). Resynthesis of IκBα in 70Z/3 cells mainly corresponds to an unphosphorylated protein that exhibits a specific interaction with NF-κB. This NF-κB/IκBα ternary complex retains NF-κB activity since, in its unphosphorylated state, IκBα is unable to mask the nuclear localization signal and DNA binding domains of the complex (22). In addition to this, unphosphorylated IκBα prevents the interaction of NF-κB with IκBα, and therefore it can contribute to a persistent NF-κB activation (23, 34).

Opposite to this situation, the translated IκBα in activated macrophages is rapidly phosphorylated, although this constitutive phosphorylation of IκBα is unsufficient to induce degradation (34). Probably for this reason, phosphorylated IκBα together with IκBα efficiently participates in the blockage and retention of NF-κB in the cytosol. In agreement with these data, only minimal amounts of IκBα were detected in the nucleus, as confirmed by different techniques including the immunolocalization by confocal microscopy, the supershift EMSA or the immunodetection by Western techniques including the immunolocalization by confocal microscopy.

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