Pervanadate-induced Nuclear Factor-κB Activation Requires Tyrosine Phosphorylation and Degradation of IκBα

COMPARISON WITH TUMOR NECROSIS FACTOR-α

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Tumor necrosis factor activates nuclear transcription factor κB (NF-κB) by inducing serine phosphorylation of the inhibitory subunit of NF-κB (IκBα), which leads to its ubiquitination and degradation. In contrast, pervanadate (PV) activates NF-κB and induces tyrosine phosphorylation of IκBα (Singh, S., Darney, B. G., and Aggarwal, B. B. (1996) J. Biol. Chem. 271, 31049–31054; Imbert, V., Rupec, R. A., Antonia, L., Pahl, H. L., Traencker, E. B.-M., Mueller-Dieckmann, C., Farahifar, D., Rossi, B., Auderger, P., Baeuerle, P. A., and Peyron, J.-F. (1996) Cell 86, 787–798). Whether PV also induces IκBα degradation and whether degradation is required for NF-κB activation are not understood. We investigated the effect of PV-induced tyrosine phosphorylation on IκBα degradation and NF-κB activation. PV activated NF-κB, as determined by DNA binding, NF-κB-dependent reporter gene expression, and phosphorylation and degradation of IκBα. Maximum degradation of IκBα occurred at 180 min, followed by NF-κB-dependent IκBα resynthesis. N-Acetylleucylleucylnorlucinal, a proteasome inhibitor, blocked both IκBα degradation and NF-κB activation, suggesting that the IκBα degradation is required for NF-κB activation. PV did not induce serine phosphorylation of IκBα but induced phosphorylation at tyrosine residue 42. Unlike tumor necrosis factor (TNF), PV did not induce ubiquitination of IκBα. Like TNF, however, PV induced phosphorylation and degradation of IκBα, and subsequent NF-κB activation, which could be blocked by N-tosyl-l-phenylalanine chloromethyl ketone, calpeptin, and pyrrolidine dithiocarbamate, suggesting a close link between PV-induced NF-κB activation and IκBα degradation. Overall, our studies demonstrate that PV activates NF-κB, which, unlike TNF, requires tyrosine phosphorylation of IκBα and its degradation.

Nuclear transcription factor-κB (NF-κB) plays a pivotal role in expression of various inducible target genes related to immune and inflammatory responses, including the type I human immunodeficiency virus (1–3). NF-κB is a dimer of members of the Rel family of proteins (1, 4). In nonstimulated cells, the heterodimeric NF-κB complexes are restricted to the cytoplasm, where they are associated with an inhibitory molecule of the IκB family (5). In mammalian species, six structural homologs of IκB have been identified (6), but only one of them, the IκBα form, has been extensively studied. IκBα regulates NF-κB activity by masking the nuclear localization signal located on the p50-p65 heterodimer of NF-κB (7, 8). In response to stimulation by various agents, among them phorbol esters (e.g. phorbol 12-myristate 13-acetate), tumor necrosis factor (TNF), interleukin-1α (IL-1α), γ-radiation, and lipopolysaccharide, IκBα undergoes degradation, allowing the p50-p65 heterodimer to migrate to the nucleus (1, 9–12). A protein kinase complex, consisting of IκB kinase α, β, and γ subunits, stimulated by TNF or IL-1 phosphorylates Ser-32 and Ser-36 of IκBα; these steps are essential for its degradation and the consequent nuclear translocation of NF-κB (13, 14). It has been demonstrated by using specific proteasome inhibitors that inducible phosphorylation of IκBα is needed but not sufficient for its degradation by proteasome (15). Before being degraded by 26 S proteasome (8, 9), serine-phosphorylated IκBα is polyubquitinated at the Lys-21 and Lys-22 positions (16, 17).

Tyrosine phosphorylation also plays a role in NF-κB activation, although that role is not fully understood. Inhibitors of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (e.g. PTPase) suppress NF-κB activation (18–23). Recently, it was shown that hypoxia, reoxygenation, and the PTPase inhibitor pervanadate (PV) induce tyrosine phosphorylation of IκBα (23–27) and activate NF-κB (23–25). Whether tyrosine phosphorylation leads to IκBα degradation and whether degradation is required for NF-κB activation are not known. In this report, we demonstrate that PV-induced tyrosine phosphorylation leads to degradation of IκBα and that this degradation is required for NF-κB activation.

EXPERIMENTAL PROCEDURES

Materials—Human myeloid U937 and epithelial HeLa cells were obtained from the American Type Culture Collection (Manassas, VA). Rabbit polyclonal antibodies anti-IκBα, anti-p50, and anti-p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against the serine-32-phosphorylated form of IκBα was purchased from New England BioLabs, Inc. (Beverly, MA). Sodium orthovanadate, pyrrolidine dithiocarbamate (PDTC), N-tosyl-l-phenylalanine chloromethyl ketone (TPCK), cycloheximide, biotinylated anti-phosphotyrosine monoclonal antibody, horseradish peroxidase-conjugated anti-biotin monoclonal antibody, anti-Flag monoclonal antibody, and the alkaline phosphatase fluorescent substrate 4-methylumbelliferyl phosphate were obtained from Sigma. Bacterium-derived recombinant human TNF, purified to homogeneity with a specific activity of 5 × 10⁸ units/mg, was kindly provided by Genentech Inc.

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(South San Francisco, CA). Calf intestine alkaline phosphatase (CIP), RPMI 1640 medium, minimum Eagle’s medium, and fetal bovine serum were obtained from Life Technologies Inc. Genistein, calpeptin, and N-acetylcysteine/l-carnosine (ALLN) were procured from Calbiochem-Novabiochem Corp. (San Diego, CA). Protein A/Sepharose beads were purchased from Pierce. The pH of the reaction mixture was neutralized with 1 N HCl, and excess H2O2 was deactivated with catalase (200 U/ml). The concentration of pervanadate generated is denoted by the vanadate concentration taken in the reaction mixture. U-937 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and a 1× antibiotic-antimycotic solution. The culture was split every 3 days. HeLa cells were maintained in MEM containing 10% fetal bovine serum.

Electrophoretic Mobility Shift Assay—NF-κB activation was analyzed by electrophoretic mobility shift assay as described previously (28). In brief, 8-μg nuclear extracts prepared from TNF- or PV-treated cells were incubated with 106 end-labeled 45-mer double-stranded NF-κB oligonucleotide for 15 min at 37 °C and the DNA complex resolved in 6.6% native polyacrylamide gel. The specificity of DNA-protein complex using specific and irrelevant antibodies. The antibody-treated samples of NF-κB were resolved on a 5.5% native gel. The reaction bands were visualized and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

Western Blot of IκBα—Thirty-microgram cytoplasmic protein extracts, prepared as described (28), were resolved on 9% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with anti-IκBα polyclonal antibodies (1:3000) for 1 h. The blot was washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h, and finally detected by chemiluminescence (ECL, Amersham Pharmacia Biotech). The bands obtained were quantitated using Personal Densitometer Scan version 1.30 using ImageQuant software version 3.3 (Molecular Dynamics, Sunnyvale, CA).

To phosphorylate IκBα, we exposed 30 μg of PV-treated cytoplasmic cell extracts to CIP (0.1–5 units) for 10 min at 37 °C. The reaction was stopped by boiling with reducing sample buffer, and the samples were subjected to electrophoresis and Western blot for IκBα.

Identification of Tyrosine-phosphorylated IκBα—After treatment with PV, cells were washed with phosphate-buffered saline, and whole cell lysates were prepared in lysis buffer (20 mM HEPES, 250 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5 mM EDTA, 0.5% EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine, 3 mM sodium orthovanadate). One milligram of lystate protein was treated with 1 μg of anti-IκBα antibodies in 800 μl of lystate buffer at 4 °C for 2 h, and the immune complexes were precipitated with protein A/G-Sepharose beads. The beads were thoroughly washed and boiled for 5 min with 1× reducing sample buffer. After boiling, the proteins were resolved on 9% SDS-PAGE gel, electrotransferred to a nitrocellulose membrane, and probed with anti-phosphotyrosine biotin monoclonal antibody (1:2000). The blot was then treated with anti-biotin-horseradish peroxidase conjugate and finally detected by ECL reagent.

Transient Transfection with IκBα Gene—Transient was performed as described earlier (26). In brief, the FLAG-tagged HeLa cells 24 h after transfection, cells were washed and incubated with complete medium without addition or containing 1 μg TNF or 200 μM PV for 24 h. Thirty micrograms of IκBα DNA was transferred to a nitrocellulose membrane, and probed with anti-FLAG antibody.

NF-κB SEAP Reporter Assay—The NF-κB-reporter gene expression assay was based on our earlier report (29). In brief, 0.5 × 106 HeLa cells/1.5 ml were plated in each well of a 6-well plate and incubated for 16–18 h. Cells were transiently transfected with pNF-κB-

SEAP2 (0.5 μg) and the expression vector (2.5 μg of pCMV) by the calcium-phosphate method for 9 h. After transfection, cells (duplicate wells) were washed and incubated with medium or with medium containing 1 ng TNF or 200 μM PV for 24 h. The culture supernatant was removed and assayed for SEAP activity. The culture supernatant (25 μl) was mixed with 50 μl of 4-methylumbelliferyl phosphate and finally treated with anti-FLAG antibody.

RESULTS

We examined the effects of PV on NF-κB and IκBα in U-937 cells because the effects on these cells are well characterized in our laboratory. The concentration and time of exposure to PV used in our experiments had no significant effect on cell viability (data not shown).

Pervanadate-induced NF-κB Activation Correlates with IκBα Degradation—We first examined the kinetics of PV-induced NF-κB activation as detected by electrophoretic mobility shift assay in U-937 cells. For this, we treated cells with 100 μM PV for different times, prepared the nuclear extracts, and analyzed them by electrophoretic mobility shift assay. Time course analysis revealed that NF-κB/DNA binding activity was first detected at 120 min, reached a maximum at 180 min, and declined thereafter (Fig. 1A). The activated form of NF-κB was evident even at 480 min. Under similar conditions, TNF-induced NF-κB activation could be noted as early as 5 min (data not shown). Thus the kinetics of NF-κB activation by PV appears to be much slower than activation by TNF.

Incubation of nuclear extracts with anti-p65 (Fig. 1B, Anti-
p65) or anti-p50 antibodies (Anti-p50) resulted in the abrogation in NF-κB/DNA complex, whereas irrelevant anti-Cyclin D1 antibodies (Anti-Cyclin D1) or preimmune sera (PIS) had no effect. Thus, NF-κB induced with PV contained both the p65 and p50 subunits. The specificity of the PV-induced NF-κB/DNA complex was further confirmed by demonstrating that the binding was disrupted in the presence of a 100-fold excess of unlabelled κB-oligonucleotide (Fig. 1B, Cold oligo) but not by mutant oligonucleotide (Mutant oligo).

Activation of NF-κB by TNF is achieved through Ser-32 and Ser-36 phosphorylation of IκBα followed by polyubiquitination and degradation, which results in the nuclear translocation of NF-κB. Whether PV-induced NF-κB activation is associated with the degradation of IκBα is not clear. To investigate this, cytoplasmic extracts from cells treated with PV for different times were subjected to Western blot analysis using IκBα-specific polyclonal antibodies. Within 30 min of PV treatment, all IκBα appeared as slow migrating species from 37 to 39 kDa, which then gradually was degraded (Fig. 1C). Maximum degradation of the slowly migrating species was noted at 240 min. Beyond 240 min, no further degradation was observed, but rather new synthesis of IκBα began. The synthesis of IκBα, which is dependent on NF-κB activation, started at 180 min (normally migrating 37-kDa size) and reached a maximum at 480 min (Fig. 1C). The kinetics of PV-induced IκBα degradation correlated well with the kinetics of activation of NF-κB (Fig. 1A). The synthesis of newly formed IκBα indicates that IκBα had been transcribed by the activated NF-κB.

NF-κB Induced by PV Is Transcriptionally Active—Due to the additional steps involved, induction of binding of NF-κB to the DNA does not always indicate transcriptional activation. Therefore, we examined the ability of PV to activate NF-κB-dependent SEAP reporter gene expression. The relative activities of SEAP

induced either by 100 μM PV or 1 nM TNF are shown in Fig. 1D. Both PV and TNF increased SEAP activity by 2.5–3-fold over control. These results indicate that NF-κB activated by PV was transcriptionally active and comparable with TNF.

Proteasome Inhibitor Blocks PV-induced IκBα Degradation and NF-κB Activation—ALLN, a peptide-aldehyde inhibitor that blocks the activity of the enzyme calpain I, is reported to inhibit the proteolytic activity of the proteasome (30) and reduce the degradation of ubiquitin-conjugated proteins (31). ALLN also blocks the TNF-induced degradation of IκBα without inhibiting its hyperphosphorylation, and this causes suppression of NF-κB activation (15). Because activation of NF-κB in PV-induced U937 cells is associated with the degradation of IκBα, we examined the effects of the proteasome inhibitor on the degradation of IκBα and subsequent NF-κB activation. As shown in Fig. 2A, ALLN blocked PV-induced degradation of IκBα (Fig. 2A, upper right panel) and attenuated NF-κB activation (Fig. 2A, lower panels). Furthermore, as indicated by the appearance of a slowly migrating band, ALLN treatment did not inhibit PV-induced hyperphosphorylation of IκBα. Similar results were obtained in case of TNF-treated cells (Fig. 2B). These ALLN results indicate that a proteasome is involved in PV-induced NF-κB activation and IκBα degradation and suggest that degradation of IκBα protein is a prerequisite for NF-κB activation.

PV Induces Synthesis of New IκBα—Cycloheximide, a protein synthesis inhibitor, completely blocks the IL-1β-induced resynthesis of IκBα (32). In the previous subsection (Fig. 1C),
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Fig. 3. Pervanadate causes Tyr-42 phosphorylation of IκBα. A, dephosphorylation of IκBα from PV-treated cells by protein phosphatase. Thirty micrograms of lysates prepared from untreated or PV-treated cells (100 μM, 30 min) was incubated with various concentrations of CIP for 10 min at 37 °C and then assayed for IκBα. B, effect of protein tyrosine kinase inhibitor (genistein) on PV-induced degradation of IκBα and activation of NF-κB. U937 cells (2 × 10⁶ cells/ml) were treated with none or 80 μg/ml genistein for 1 h, followed by induction with 100 μM PV for the indicated times. Cytoplasmic extracts were analyzed for IκBα (upper panels) and nuclear extracts for NF-κB (lower panels). s, slowly migrating band; n, normally migrating band. C, anti-phosphotyrosine Western blot (WB) analysis of IκBα immunoprecipitated from PV-treated cells. IκBα was immunoprecipitated (IP) from untreated (control) or PV-treated cells either by preimmune sera (PIS) or anti-IκBα antibodies and protein A/G-Sepharose and immunoblotted with biotinylated anti-phosphotyrosine antibody (left two panels) as described under “Experimental Procedures.” The same blot was reprobed with anti-IκBα antibodies (right two panels). D, cell transfection of FLAG-tagged IκBα implicates Tyr-42 as the phosphorylation site induced by PV in vivo. HeLa cells were transfected with pCDNA3, epitope-tagged Y42F mutant IκBα, or wild type (WT) IκBα as described under “Experimental Procedures.” Cells were left untreated or treated with 100 μM PV for 2 h, whole cell lysates were prepared, and 30 μg of protein was resolved on 10% SDS-PAGE gel and immunoblotted with anti-FLAG antibody as described under “Experimental Procedures.” E, we have shown that upon PV treatment, IκBα was first degraded and then resynthesized in U937 cells. To reconfirm this, we treated the cells with PV for different times in the presence of cycloheximide and then examined the cellular IκBα levels. Cycloheximide caused complete cessation of IκBα synthesis, as no IκBα was detected after 60 min (Fig. 2C). The inhibition of degradation by proteasome inhibitor and blockage of resynthesis by cycloheximide indicates that IκBα resynthesis is mediated through the activation of NF-κB (Fig. 1C).

PV-induced Slowly Migrating Band Is Due to Tyrosine Phosphorylation of IκBα—To verify that the slowly migrating species of IκBα that appeared after PV treatment was due to its phosphorylation, cytoplasmic extracts from PV-treated cells were incubated with different concentrations of CIP. To facilitate the dephosphorylation reaction, a vanadate-free lysis buffer was used. As shown in Fig. 3A, incubation with CIP did not affect the normally migrating band (37 kDa) of IκBα, but the slowly migrating band was completely converted to the 37-kDa IκBα noted in control untreated cells. This demonstrates that IκBα was phosphorylated by PV.

To determine whether slow migration was due to phosphorylation of IκBα, U937 cells were incubated with genistein, a PTK inhibitor, for 1 h and then exposed to PV. Genistein completely blocked PV-induced IκBα phosphorylation and degradation, and this correlated with total suppression of NF-κB activation (Fig. 3B). These data suggest that a genistein-sensitive PTK is activated by PV to phosphorylate IκBα and this phosphorylation is needed for IκBα degradation and subsequent NF-κB activation.

PV Induces Phosphorylation of IκBα at Tyrosine 42—Metabolic inhibitors such as genistein are not always specific. We and other groups have shown that PV induces phosphorylation of IκBα at tyrosine 42 (25–27). To confirm this in our system, we first investigated tyrosine phosphorylation of IκBα by immunoprecipitation and Western blot analysis. Untreated and PV-induced whole cell lysates were immunoprecipitated either with preimmune sera or with anti-IκBα antibodies followed by Western blot with the anti-phosphotyrosine antibody. As shown in Fig. 3C (left two panels), IκBα immunoprecipitated from PV-treated cells, but not from control untreated cells, was indeed tyrosine-phosphorylated. The tyrosine-phosphorylated band was confirmed to be the slower migrating species (39 kDa) of IκBα by Western blot analysis using anti-IκBα antibodies (Fig. 3C, right two panels).

Next, we transfected FLAG-tagged wild type and mutant (Y42F) IκBα genes into HeLa cells and analyzed the expression of IκBα protein in control (untreated) and PV-treated cells by Western blot with anti-FLAG antibody. The FLAG-tagged Y42F-IκBα did not become phosphorylated and migrated faster than the wild type IκBα in SDS-PAGE gel (Fig. 3D). Furthermore, FLAG-tagged wild type IκBα decreased in mobility upon PV treatment, providing evidence that in our system, PV induced phosphorylation of IκBα at Tyr-42 in vivo.

PV Does Not Phosphorylate at Ser-32 of IκBα—TNF-induced phosphorylation of IκBα at Ser-32 and Ser-36 is essential for its...
degradation and subsequent NF-κB activation (14, 33). To study whether IκBα phosphorylation at these sites was required for degradation induced by PV, ALLN-pretreated U937 cells were induced with either TNF or PV. The cytoplasmic extracts were probed with Ser-32 phosphospecific IκBα antibody. As shown in Fig. 3E, neither untreated nor ALLN-treated cells showed any Ser-32-phosphorylated IκBα species. Upon TNF treatment, however, phosphorylated IκBα began to appear as early as 5 min (Fig. 3E, left panel). No Ser-32-phosphorylated IκBα was detected in PV-treated cells. Reprobing the same blot with anti-IκBα antibodies verified the presence of IκBα (Fig. 3E, right panel). These data suggest that unlike...
cystine induction, PV-induced IkB degradation is not due to serine phosphorylation.

**PV Does Not Induce Ubiquitination of IkBα—TNF/IL-1-induced degradation of IkBα requires phosphorylation at Ser-32 and Ser-36 followed by polyubiquitination at Lys-21 and Lys-22 (16, 17). To examine whether PV induces ubiquitination of phosphorylated IkBα before degradation, we treated U937 cells with ALLN and then with either 0.1 mM TNF for 3, 7, and 10 min or 100 μM PV for 15, 30, and 60 min. Cytoplasmic extracts (60 μg of protein) were resolved and probed with anti-IkBα antibodies. As shown in Fig. 3F, a ladder of high molecular mass proteins appeared following stimulation with TNF, and the signals intensified at 10 min. The molecular mass increments of this ladder were ~8.5 kDa, which is the size of ubiquitin. In contrast, PV-treated samples did not show any such ladder. This result suggests that PV does not induce IkBα ubiquitination. To enhance the detection limit for ubiquinated IkBα protein, the blot was exposed for a longer duration, resulting in broader bands of IkBα. Equally intensified nonspecific band in each lane signifies equal loading of the samples.

**PDTC Blocks Tyrosine Phosphorylation and Degradation of IkBα—**Numerous reports have demonstrated that cytokine-induced NF-κB activation is sensitive to intracellular redox changes. The oxygen radical scavenger PDTC has been reported to block signal-induced phosphorylation of IkBα and its degradation, leading to suppression of NF-κB activation (34, 35). PDTC was also reported to prevent PV-induced cleavage of ErbB-4 (36). We therefore examined the effect of PDTC on PV-induced degradation of IkBα and on activation of NF-κB. U937 cells were treated with PDTC prior to induction with either TNF or PV. As shown in Fig. 4A, PDTC effectively blocked both TNF- and PV-induced IkBα degradation and NF-κB activation. In addition, PDTC also blocked both PV- and TNF-induced phosphorylation of IkBα.

**Serine Protease Inhibitor Blocks IkBα Degradation and NF-κB Activation—**TNF-induced activation of NF-κB is a result of sequential events, such as phosphorylation, polyubiquitination, and finally degradation of IkBα by the 26 S proteasome (4). We have shown in the previous section that ALLN, a proteasome inhibitor, suppresses PV-induced IkBα degradation and NF-κB activation. Here, we investigated the possibility of the involvement of other proteases in the degradation of IkBα in U937 cells. The serine protease inhibitor and alkylation agent TPCK, reported to be an effective inhibitor of IkBα degradation and NF-κB activation (38, 39). In our studies, TPCK completely prevented TNF-induced IkBα degradation and NF-κB activation (Fig. 4B, upper panels). Although TPCK completely blocked PV-induced NF-κB activation, it did not completely protect PV-induced degradation of IkBα (Fig. 4B, lower panels). Unlike ALLN, however, TPCK also blocked IkBα phosphorylation induced by both TNF and PV.

**Calpain Protease Inhibitor Also Blocks IkBα Degradation and NF-κB Activation—**Next, we tested calpeptin, an inhibitor of calpains, a group of cytosolic Ca2+-activated thiol proteases that are implicated in TNF-induced IkBα degradation (40–42). The result shown in Fig. 4C indicates that IkBα degradation was induced by both PV and TNF and that calpeptin treatment significantly blocked the degradation. Calpeptin also prevented phosphorylation of IkBα induced by both TNF and PV. This correlated with suppression of TNF- or PV-induced NF-κB activation by calpeptin (Fig. 4C, lower panels). These results suggest that PV induces degradation of IkBα by activating protease similar to the one activated by TNF. These results also suggest that suppression of IkBα degradation blocks PV-induced NF-κB activation.

**DISCUSSION**

In this report, we studied how PV induces NF-κB activation in U937 cells. Our results showed that PV-induced NF-κB activation was associated with IkBα degradation. PV-activated NF-κB was functional, as it induced IkBα resynthesis and activated reporter gene expression. Results of genistein sensitivity, immunoprecipitation, Western blot, and site-specific mutagenesis experiments revealed that PV phosphorylated the Tyr-42 residue of IkBα, the Ser-32 residue remained unphosphorylated, and IkBα is not ubiquitinated before degradation. Although PV induces tyrosine phosphorylation of IkBα and TNF induces serine phosphorylation, various inhibitors, such as ALLN, PDTC, TPCK, and calpeptin, which work through different mechanisms, all blocked IkBα degradation and NF-κB activation induced by PV. These results indicate that the degradation of IkBα is required for NF-κB activation.

Previously, it was reported that PV induces NF-κB activation without degrading IkBα (25). Another report indicated that the p85α subunit of phosphotyidylinositol 3-kinase binds to the PV-induced tyrosine-phosphorylated IkBα and dissociates it from the p50-p65 complex (27). Our results, however, indicate that PV induces tyrosine phosphorylation of IkBα, which in turn leads to its degradation. Why there is a difference between our results and those reported by Imbert et al. (25) is not clear. The difference in results may be due to the difference in kinetics of NF-κB activation: Imbert et al. (25) reported maximum activation of NF-κB when Jurkat (T cells) cells are treated with 250 μM PV for 60 min, whereas we found optimum activation when cells were treated with 100 μM PV for 180 min. Similarly, Imbert et al. (25) monitored IkBα levels maximally up to 150 min after treatment of cells with 200 μM PV and found no significant degradation of IkBα. We found that 100 μM PV-induced IkBα degradation begins at 30 min and reaches a maximum at 240 min (see Fig. 1C). Besides kinetics, the difference may also be due to cell type (myeloid versus T cells) used.

Another important difference between our results and those of Imbert et al. (25) is that the latter workers noted no resynthesis of IkBα after PV treatment. Our studies, however, clearly show that the resynthesis of IkBα began at 180 min and reached its starting level 480 min after PV treatment (see normally migrating band in Fig. 1C). As resynthesis is dependent on NF-κB activation, we found that the latter precedes the resynthesis. The effect of cycloheximide on the suppression of resynthesis induced by PV can be noted as early as 120 min after treatment (see Fig. 2C). Imbert et al. (25) found a partial degradation of phosphorylated IkBα at 150 min only when cycloheximide-pretreated cells were exposed to PV. The normally migrating 37-kDa band of IkBα was not induced by PV in studies reported by Imbert et al. (25).

In agreement with previous results (25–27), our studies clearly demonstrate that PV induces phosphorylation of IkBα at tyrosine residue 42. Our studies also suggest that tyrosine phosphorylation is required for IkBα degradation. We found that treatment of cells with various metabolic inhibitors with diverse mechanisms of action blocked IkBα phosphorylation and degradation simultaneously. ALLN, a proteasome inhibitor, however, blocked PV-induced degradation without blocking tyrosine phosphorylation of IkBα, indicating that phosphorylation alone is not sufficient to induce degradation. Genistein (a PTK inhibitor), PDTC (an inhibitor of certain metalloproteases and reactive oxygen intermediate quencher), TPCK (a serine protease inhibitor), and calpeptin (a calpain inhibitor) suppressed both phosphorylation and degradation of IkBα. These results clearly suggest that phosphorylation of IkBα is needed but not sufficient for degradation induced by PV. This is consistent with results reported for cytokine-induced IkBα degra-
Phosphorylation of Tyr-42 in IκB is in accord with previous findings (25, 26). Our result on anti-Ser32-phosphorylated IκB Western blot confirms that PV did not phosphorylate cytokine-inducible Ser32/Ser36 sites of IκBα. Treatment of Jurkat cells with PTKC prevents tyrosine phosphorylation by peroxovanadium compounds (37), as it did in our observations. Because phosphorylation precedes the degradation of IκBα, it is apparent that these compounds somehow block the action of PTK rather than inhibit the proteolysis of IκBα. Because PTKC prevented IκBα phosphorylation, reactive oxygen intermediates could be inducers of tyrosine phosphorylation of IκBα. This result supports previous observations of reversible phosphorylation of IκBα upon reoxygenation of Jurkat cells (25). Mammalian cells do not produce large amounts of antioxidant enzymes during hypoxia. As a result, reactive oxygen intermediates are immediately generated upon reoxygenation, which leads to tyrosine phosphorylation of various regulatory proteins. H2O2 is reported as a potent inhibitor of PTPase that may lead to the activation of PTKs and thereby to tyrosine phosphorylation (47). How calpeptin prevents tyrosine phosphorylation is not clear. Our results with calpeptin, the inhibitor of thiolyte protease (cytosolic calpain), and proteasome inhibitor (ALLN) probably indicate that tyrosine-phosphorylated IκBα is degraded by both cytosolic calpain-calpastatin and ubiquitin-proteasome pathways, similar to that reported for TNF-induced degradation of IκBα (42). Overall, our results suggest that PV-induced tyrosine phosphorylation leads to the degradation of IκBα and the activation of NF-κB in U937 cells. These results may be relevant to physiological stimuli, such as anoxia, that activate NF-κB through tyrosine phosphorylation.

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