Tyrosine Nitration on p65
A NOVEL MECHANISM TO RAPIDLY INACTIVATE NUCLEAR FACTOR-κB

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NO is an important factor that induces post-translational modifications of proteins by cellular reduction and oxidation mechanism: cysteiny1-nitrosylation or Tyr nitration. Nuclear factor (NF)-κB activity can be rapidly suppressed by sodium nitroprusside, a NO donor. This effect was effectively reversed by peroxynitrite scavenger deferoxamine, suggesting a Tyranitration-mediated mechanism. Western blot with nitrotyrosine-specific antibody demonstrated that the p65 subunit of NF-κB was predominantly nitrated on Tyr residues. Tyr nitration of p65 induced its dissociation from p50, its association with IκBα, and subsequent sequestration of p65 in the cytoplasm by IκB-mediated export. Liquid chromatography-coupled nanoelectrospray mass spectrometry revealed specific nitration on Tyr-66 and Tyr-152 residues. Alterations of IκBα substrate-specific protein kinase. In addition, NO generated by inducible NO synthase or administered exogenously can directly modify target proteins through NO-dependent protein kinase. Numerous studies have examined the mechanisms underlying NF-κB activation. However, it is less clear whether and how NF-κB can be rapidly and efficiently inactivated by other post-translational modifications.

NF-κB is an inducible transcription factor that regulates the expression of a wide variety of genes. It is a key regulatory factor for various cellular events, including cell survival, cell proliferation, angiogenesis, and metastasis of cancers (1–4). NF-κB generally exists as homo- or heterodimers of five family members including p50/p105 (NF-κB1), p52/p100 (NF-κB2), c-Rel, RelB, and p65 (RelA).

In most cells, NF-κB is predominantly cytoplasmic and silent because of its association with an inhibitory protein of the IκB family, IκBα, -β, -ε, or Bcl-3. The p50 or p52 homodimer binds to the κB DNA element to repress target gene expression by recruiting nuclear receptor corepressors and histone deacetylases to the target gene promoter (5–8). Upon stimulation by proinflammatory cytokines, the bacterial cell surface antigen lipopolysaccharide, viral infection, phospholipase, or radiation, IκBα is phosphorylated by IκB kinase, ubiquitinated, and degraded by a 26 S proteasome protease complex. The dissociated NF-κB p65/p50 heterodimer is then translocated into nuclei to activate target gene expression (9). Translocation into nuclei is considered to be a general activating mechanism for NF-κB. Recently post-translational modifications of p65 have been reported, including phosphorylation and acetylation (7, 10–15). These post-translational modifications affect the association of p65 with transcriptional coactivators, such as CBP/p300, or the inhibitory molecules such as IκBα or histone deacetylase 3. Numerous studies have examined the mechanisms underlying NF-κB activation. However, it is less clear whether and how NF-κB can be rapidly and efficiently inactivated by other post-translational modifications.

NF-κB activity is regulated by various factors, including NO. NO is a well known signaling molecule involved in pathophysiological processes such as inflammation, apoptosis, regulation of enzyme activity, and gene expression. NO triggers the cGMP second messenger system, as originally identified in vascular smooth muscle (16), that activates the cGMP-dependent protein kinase. In addition, NO generated by inducible NO synthase or administered exogenously can directly modify target proteins through S-nitrosylation of cysteine residues or nitration of Tyr residues (17, 18). Tyr nitration is caused by peroxynitrite generated from NO and superoxide and is implicated in apoptosis and degenerative diseases. NO can exert anti-inflammatory effects through inhibiting NF-κB activity. Two mechanisms are known to mediate NO suppression of NF-κB activity: one is through S-nitrosylation of the p50 subunit, which results in reduced DNA binding activity (19, 20), and the second is by S-nitrosylation of IκB kinase β to reduce IκBα phosphorylation (21, 22). Both repressive mechanisms would probably be less efficient once NF-κB has been activated. However, in most cancer cells NF-κB is constitutively active and associated with DNA, and hence, the suppressive effect of NO by S-nitrosylation of the p50 subunit or the IκB kinase β protein would seem to be inefficient. In our previous studies (23), we found NO rapidly and efficiently suppressed c-myc by inactivating NF-κB in P19 carcinoma...
cells where NF-κB was constitutively active and peroxynitrite scavenger restored c-myc expression. Similarly NO impaired DNA binding of the NF-κB p65/p50 heterodimer activated by tumor necrosis factor (TNF)-α in HEK293 cells, which was also abolished by peroxynitrite scavenger. The DNA binding of p50 homodimer was not affected. These raised the possibility that NO might inactivate the readily active NF-κB through alternative mechanisms.

The differential effect of NO on the p65/p50 heterodimer (active NF-κB) versus the repressive p50 homodimer suggested that p65 could be the target of the rapid suppressive effect of NO. This observation prompted us to examine an alternative peroxynitrite-triggered mechanism, Tyr nitration on p65. We demonstrated by subcloning IκB into pCMV with a TRIzol® kit (Invitrogen). The endogenous human genes. Using liquid chromatography-coupled nanoelectrospray ionization mass/mass spectrometry (LC/MS/MS), we identified two specific Tyr residues of p65 that could be nitrated: Tyr-66 and Tyr-152. Both residues were located in the Rel homology domain of p65. Mutation studies confirmed the role of these two Tyr residues in p65 activity. We now report the studies identifying a novel NO-triggered rapid “off-switch” for NF-κB by inducing nitration on two specific Tyr residues of p65.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—pEGFP-c1-p65 was constructed by fusing p65 to the C terminus of EGFP (Clontech). The point mutants Y66F and Y152F were constructed from the pairs of point-mutated PCR primers and pEGFP-c1-p65 as a template plasmid by PCR methods using the QuikChange® XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. pcDNA-IκBα was constructed by subcloning IκBα (24) into pcDNA3.1 (Invitrogen).

**Cell Culture and Reporter Assay**—P19, COS-1, and HEK293 were maintained as described previously (25). SH-SY5Y cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. P19 cells were transiently transfected with a NF-κB reporter vector, pGL4-Luc, and the sizes (in parentheses) of expected PCR products were as follows: hACTB (873 bp); mlCAM-1, 5'-GCTCTGGCCCTGTCACCGTG-3' and 5'-GAGTTTTATGGCCTCCTCGGAG-3' (1502 bp); and human actin-β1 (as an internal control), 5'-GGTCAACAGGCTCCGCAT-G-3'and 5'-GGTGCCAGATTTTCTCAGTGC-3' (236 bp). PCRs were performed at annealing temperatures from 55 to 60 °C with 27–35 cycles.

**Chromatin Immunoprecipitation (ChIP)**—P19 cells were treated with SNP (0–6 h), and HEK293 cells were treated with TNF-α for 1 or 2 h prior to SNP treatment (0–90 min). ChIP assays were performed (23) using 2 μg each of anti-p65 (H-286, Santa Cruz Biotechnology, Inc.), anti-p50 (06-886, Upstate Biotechnology), or preimmune serum (Pierce, 31884). Precipitated DNA was amplified by PCR with the following primers specific to the xB flanking sequences of the endogenous mlCAM-1 or hICAM-1 promoter: mlCAM-1, 5'-TGTGTCTCTGACAACCCTCC-3' and 5'-TAGCAGGTGAGACTCTGA-3'; hICAM-1, 5'-GATTCAAGCTTAGCCTGGCCGG-3' and 5'-GAGTACGCAAGGACTCTGGC-3'.

**Immunoblotting, Immunoprecipitation, and in Vitro Protein Interaction Assay** (His Pull-down)—Nuclear, cytoplasmic, or whole cell extracts were subjected to immunoblotting and/or immunoprecipitation using antibodies as described in each figure legend. IκBα was synthesized using an in vitro transcription and translation kit (Promega, Madison, WI). His-tagged p65 was precipitated with nickel-nitrilotriacetic acid (Ni-NTA)-agarose beads (Qiagen, Valencia, CA) and allowed to interact with 32P-labeled IκBα. Protein complexes were resolved by SDS-PAGE and immunoblot analyses.

**In Vitro Nitration and LC-MS/MS Analysis of p65**—P19 whole cell extracts or recombinant p65 (GenBank™ accession no. AAA36408) (TransAM™ NFκB p65, Active Motif, Carlsbad, CA) were incubated in a nitrogen reaction buffer containing 10 mM NaNO₂, 9 μM FeCl₃, 0.3% H₂O₂, and 20 mM sodium acetate (pH 5.6) for 24 h at room temperature followed by immunoblotting or immunoprecipitation using anti-NTyr. BSA was included as the in vitro nitration control (26).

Four hundred nanograms of nitrated or unmodified recombinant p65 were digested with trypsin (Promega) using PrePro (Genomic Solutions, Ann Arbor, MI). After trypsin digestion, 20 μl of the un-nitrated p65 sample was diluted to a final volume of 30 μl in 98:2 water:6-aminoquinacridone, 0.1% 1% trifluoroacetic acid. Nitrated sample was analyzed without dilution. The peptide solution (27.5 μl) in a loading buffer (98:2 water:6-aminoquinacridone and 0.1% formic acid) was aspirated by an LC Packings (Dionex, Sunnyvale, CA) Famos autosampler, and peptides were eluted at 350 nl/min using an LC Packings Ultimate LC system. This LC system is equipped with a C18 particle column packed with 5-μm stationary phase, 12-cm capillary column packed with 5-μm and 200-Å pore size C₈ particles (Michrom BioResources, Auburn, CA). Peptides were eluted with a 1.6% solvent B/min linear gradient over 65 min starting with 100% solvent A (where solvent A was 95:5 water:6-aminoquinacridone, 0.1% formic acid and solvent B was 5:95 water:6-aminoquinacridone, 0.1% formic acid). The LC system was on line with an Applied Biosystems (ABI, Foster City, CA) QSTAR Pulsar quadrupole TOF MS system, which was equipped with a Protea nanoelectrospray source. As peptides eluted from the column they were focused into the mass spectrometer where precursor ion spectra were collected in an information-dependent acquisition mode. Information-dependent acquisition mode settings included continuous cycles of one full TOF MS scan from 400–1100 m/z (1.5 s) plus three product ion scans from 50–2500 m/z (3 s each). Precursor m/z values were selected from a peak list automatically generated by Analyst QS software (ABI) from the TOF MS scan during acquisition, starting with the most intense ion.

Peptide Mass software (Swiss Institute of Bioinformatics; available online via the ExPASy Molecular Biology Server (www.expasy.org)) was used to generate a theoretical tryptic digest of p65, and 44.881
Da was added to the mass of Tyr-containing peptides to account for possible nitrification. From the LC/MS data, the molecular weights of the detected peptides were calculated using the LC/MS reconstitute feature of Analyst QS. Experimentally measured peptide masses were compared with the theoretical digest, and to confirm the sequence of the peptides and the sites of modification MS/MS spectra were examined using the Sequence Peptide feature of Analyst QS with the following settings: m/z tolerance, 175 ppm; minimum peak height, 3%.

Analysis of p65 Export by Fluorescence Microscopy—HEK293 cells were transfected with pEGFP-c1-p65 plasmid and treated with 10 ng/ml TNF-α for 2 h followed by 0.5 mM SNP or 0.1 mM peroxynitrite for 2 h. Twenty nanomolar leptomycin B (LMB) or 10 μM DFO were added for 2 h or 30 min, respectively, prior to SNP treatment. The cytoplasmic or nuclear green fluorescent protein (GFP)-p65 was scored among at least 200 transfected cells in multiple microscopic fields and photographed using an inverted fluorescence microscope (TE200, Nikon).

**RESULTS**

Peroxynitrite Modulates the Expression of NF-κB Target Genes—The time course of NO effects on NF-κB target genes was examined with reverse transcription-PCR. In P19 and SH-SY5Y cells where NF-κB was constitutively active, SNP suppressed mICAM-1 and cyclin D1 genes (Fig. 1, A and B) within 30–120 min. In HEK293 cells where NF-κB was stimulated by TNF-α, SNP also rapidly suppressed the expression of hICAM-1, cIAP-1, and IL-8 genes (Fig. 1C). These results showed that SNP indeed effectively and rapidly inactivated NF-κB. To determine whether the rapidly suppressive effect of NO on NF-κB activity could be mediated by peroxynitrite, we examined the expression level of several endogenous NF-κB target genes in the presence of NO. The constitutively active NF-κB target genes mICAM-1 and cyclin D1 were suppressed by SNP, a direct NO donor (Fig. 1, D and E). DFO, a peroxynitrite scavenger, blocked NO-triggered suppression of mICAM-1 gene expression, whereas DTT and ODQ, an S-nitrosylating inhibitor and a guanylate cyclase inhibitor, respectively, had little or slight effect (Fig. 1, D and E). These results suggest that peroxynitrite may be primarily involved in NF-κB inactivation.

We then examined the effects of NO on TNF-α-stimulated NF-κB activity. SNP also suppressed the cIAP-1 and hICAM-1 genes induced by TNF-α-activated NF-κB in HEK293 cells (Fig. 1F). Again the NO-induced suppression of these genes was rescued by DTT and DFO but not by ODQ. Without stimulation by TNF-α, the expression of these genes was hardly affected by SNP (data not shown). Taken together, SNP suppressed all the NF-κB target genes examined through peroxynitrite regardless of the type of NF-κB activation (constitutive or stimulated). To confirm the specific effects of peroxynitrite, P19 cells transfected with His-p65 or a control were treated directly with peroxynitrite, and reporter assays were performed (Fig. 1G). As expected, cotransfection with His-p65 dramatically increased the relative luciferase activity. Both SNP and peroxynitrite effectively suppressed the transcriptional activity of His-p65. These results suggested that NO effectively suppressed NF-κB target genes via peroxynitrite by inactivating both constitutively active and cytokine-activated NF-κB.

**Tyr Nitration of p65 Subunit**—Because peroxynitrite was known to trigger Tyr nitration, immunoprecipitations were performed in P19 cell extracts treated with SNP to determine the nitrated subunit of the endogenous NF-κB (Fig. 2A). The level of Tyr nitration (α-Ntyr) was abundant on p65 (upper panel, lane 2) and much less abundant on p50 (lower panel, lane 2). As predicted, Tyr nitration was completely blocked by DFO.
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Fig. 2. Tyrosine nitration of the p65 subunit of NF-κB. A, P19 cells were pretreated with DFO for 30 min prior to SNP treatment for 6 h. Immune complexes were precipitated with antibodies against p65 (upper panel) or p50 (lower panel) and detected on immunoblots with anti-NTyr antibody. B, whole cell extracts (WCE) of COS-1 cells transfected with His-p65 and treated with SNP for 6 h were precipitated with nickel beads. The precipitates were immunoblotted with anti-p65 (lanes 2 and 3) or anti-NTyr antibodies (lanes 4 and 5). Con, control.

Fig. 3. NO-induced subunit exchange on the mouse and human ICAM-1 gene promoter. A, P19 cells were treated with 0.5 mM SNP for 0–6 h. ChIP assays were performed with antibodies specific to p65 and p50, and DNA was amplified with primers specific to the mICAM-1 gene promoter and probed. B, HEK293 cells were pretreated with 10 ng/ml TNF-α for 0–2 h prior to treatment with 0.5 mM SNP for 0–90 min. ChIP assays were performed with antibodies, and DNA was amplified with primers specific to the hICAM-1 gene promoter. Con, control.

Effects on NF-κB Heterodimerization and Interaction with IκBα—To directly examine whether Tyr nitration affected heterodimerization of NF-κB, immunoprecipitations were performed (Fig. 4A). Immune complexes precipitated with anti-p65 or anti-p50 antibodies were probed reciprocally with anti-p50 or anti-p65 antibodies. The results clearly demonstrated decreased heterodimerization after nitration of p65 (lane 3 versus lane 4), despite similar input levels (lanes 1 and 2). To monitor its interaction with IκBα, His-p65 was precipitated with nickel beads and incubated with [35S]-labeled IκBα followed by extensive washing. Anti-His antibody showed a similar amount of His-p65 precipitate (Fig. 4B, top panel, lanes 3 and 4). Precipitates from cells exposed to SNP showed significant Tyr nitration of His-p65 (second panel, lane 4). Although IκBα interacted moderately with the unmodified p65 (lower panel, lane 3), it interacted even more strongly with the nitrated p65 (lower panel, lane 4). Taken together, these results confirmed that Tyr nitration of p65 triggered its preferential interaction with IκBα over p50, leading to the formation of suppressive complexes.

SNP-triggered p65 Export to the Cytoplasm with IκBα—Since nitrated p65 preferentially interacted with IκBα, we then determined whether cellular localization of p65 was affected by SNP treatment. As expected, p65 was more abundant in the nuclei than in the cytoplasm of P19 control cells, consistent with the constitutive activation of NF-κB in P19 cells (Fig. 5A, top panel, t = 0). SNP dramatically reduced its nuclear...
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Increased cytoplasmic p65 by about 20% (E and L). Most p65 was translocated into the nuclei upon TNF-α stimulation (F) but relocalized into the cytoplasm following SNP treatment (G). Activated p65 was retained in the nuclei by treatment with LMB (H). Furthermore LMB completely blocked the export of p65 induced by SNP (I), supporting the existence of an IκBα-mediated export mechanism for p65 in cells treated with SNP. As predicted, DFO also blocked the export of p65 induced by SNP (J and K). These results confirmed that, in addition to reducing p65/p50 heterodimerization, Tyr nitration of p65 also facilitated its nuclear export together with the inhibitory IκBα.

p65 Nitration at Tyr-66 and Tyr-152—To determine which Tyr residues of p65 were nitrated, cell lysates from P19 cells were subjected to in vitro nitration and immunoprecipitated with anti-p65 antibody followed by immunoblot using anti-NTyr (Fig. 6A, lanes 2 and 3) or anti-p65 to monitor the input (lanes 4 and 5). BSA was used for the control of in vitro nitration (lane 1) (26). Similarly the recombinant p65 (Gen-BankTM accession no. AAA36408) was subjected to a parallel in vitro nitration and detected with anti-NTyr (Fig. 6B, lanes 2 and 3). As shown, both the endogenous and the recombinant p65 were effectively nitrated. To rule out the possibility of random nitration, purified ligand binding domain of retinoid X receptor, which contains four Tyr residues, was also subjected to in vitro nitration and detected with anti-NTyr (Fig. 6B, lanes 6 and 7) or anti-retinoid X receptor (lanes 8 and 9). This control experiment showed absolutely no nitration on this irrelevant protein and further confirmed that p65 could be nitrated in vitro specifically.

The specifically nitrated Tyr residues were then identified with mass spectrometry. The nitrated recombinant p65 (Fig. 6B, lanes 2 and 3) and the control p65 protein (lane 4) were subjected to trypsin digestion followed by LC/MS/MS. The 44.9-Da difference in precursor molecular mass between the two ions was consistent with the addition of a nitro group and loss of a hydrogen atom (14). As shown in Fig. 6C, amino acid mapping of this peptide by MS/MS analysis clearly demonstrated a 44.9-Da increase (lower panel) from the un-nitrated peptide (upper panel), consistent with nitration of the peptide. Singly charged y7 ions from both nitrated and control peptides had a mass of 687.41 Da, but the masses of singly charged y8, y9, and y10 ions all showed an increase of 44.9 Da in the nitrated peptide. In addition, the 181.07-Da peak confirmed the presence of the immonium ion of NTyr, whereas the 136.10-Da Tyr immonium ion was detected from the un-nitrated precursor. Also the shift from 335.14 to 380.14 Da indicated the conversion of the internal fragment NGY (residues 64–66) to NGY-NO2, confirming nitration at Tyr-66 of a peptide corresponding to residues 63–73 with sequence INGYTGPVTG (Fig. 6C, upper and lower panels).

Similarly Fig. 6D shows amino acid mapping of the peptide corresponding to amino acids 150–158 with sequence GYD-DLNAVR. Peak y6 from both the nitrated and control peptides had a mass of 687.41 Da, but the mass of y7 increased 44.9
Da in the nitrated peptide (upper and lower panels). Peak b2 from both nitrated and control peptides had a mass of 173.06 Da, but the masses of peaks b3 and b4 also showed an increase of 44.9 Da in the nitrated peptide. The NTyr immo-nium fragment was detected at 181.07 Da, which confirmed nitration of Tyr-152. This mass spectrometry analysis clearly confirmed that two tyrosine residues (Tyr-66 and Tyr-152), among a total of 11 tyrosine residues, were specifically and strongly nitrated. Both Tyr-66 and Tyr-152 are located in the Rel homology domain (RHD), spanning residues 19–306, which suggests a potential biological significance.

Nitration at both Tyr-66 and Tyr-152 Is Important for p65 Export and Activity—Mass spectrometry revealed two nitrated Tyr residues in RHD of p65 (Fig. 7A). To determine whether these residues were important for p65 property or activity, we constructed point mutations at Tyr-66 or Tyr-152 on a GFP-fused p65. HEK293 cells were transfected with wild type or mutant p65 and treated with TNF-α and peroxynitrite (Fig. 7B). Consistent with the result of SNP treatment (Fig. 5L), peroxynitrite also induced export of the wild type p65 (Fig. 7B, wt column). Nuclear retention of these two point mutants, Y66F and Y152F, was slightly less efficient than the wild type. Interestingly peroxynitrite could no longer induce their export effect effectively. To further examine the effects of mutation on p65 biological activity, a reporter assay was conducted. In this assay, both mutants also exhibited a lower transcriptional activity than the wild type (Fig. 7C) consistent with the less efficient nuclear retention observed for these mutated p65 proteins (Fig. 7B). The wild type p65 was significantly affected by peroxynitrite treatment (45%), whereas both mutants were
FIG. 6. Nitration of the p65 subunit at Tyr-66 and Tyr-152. A, immunoblot analysis of P19 cell extracts nitrated in vitro and immunoprecipitated (IP) with anti-p65 followed by immunoblotting (IB) with anti-NTyr (left panel) or p65 (right panel). BSA was used as a nitration control (lane 1). NO, nitrated samples. Con, untreated controls. B, recombinant p65 was nitrated in vitro. One to ten nanograms of the sample were probed on immunoblots with anti-NTyr antibody (lanes 2 and 3). Lane 4 is unmodified control p65. As a negative nitration control, purified ligand binding domain of retinoid X receptor (RXR) was nitrated in vitro followed by immunoblots with anti-NTyr (lanes 6 and 7) or anti-retinoid X receptor (lanes 8 and 9). C, LC/MS/MS spectrograph of the ionized peptides derived from tryptic digests of p65. The peptide was sequenced as INGYNO2TGPGTVR (residues 63–73). Upper spectrum, control peptide (doubly charged at m/z 567.81). Lower spectrum, in vitro nitrated peptide (doubly charged at m/z 590.31). The peaks y8*, y9*, y10*, and a4-NH3* from nitrated samples exhibit a shift of 44.9 Da relative to those of controls. NGY-NO2, nitrated Tyr-66 peptide fragment. #, immonium ion of nitrotyrosine. D, LC/MS/MS spectrograph of the ionized peptides derived from tryptic digests of p65. The peptide was sequenced as GDYN02DLNAVR (residues 150–158). Upper spectrum, control peptide (doubly charged at m/z 511.81). Lower spectrum, in vitro nitrated peptide (doubly charged at m/z 534.32). The peaks y7*, b3*, and b4* from nitrated samples exhibit a shift of 44.9 Da relative to those of controls. #, immonium ion of nitrotyrosine.
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**DISCUSSION**

NF-κB has attracted an enormous amount of attention because of its role in a wide variety of cellular processes, particularly the immune and stress responses, and in diseases. The activating and inhibitory mechanisms of NF-κB have been widely investigated to determine potential targets for drug intervention. Several post-translational modifications on components involved in NF-κB activity are known. Our study presents an additional, perhaps more general, inhibitory signal to rapidly inactivate NF-κB by Tyr nitration on the p65 subunit. While multiple NO-triggered events are involved in the regulation of NF-κB, the mechanism mediated by Tyr nitration on p65 can certainly provide a very rapid off-switch for readily active NF-κB upon peroxynitrite formation. Nevertheless our results could not rule out suppression by S-nitrosylation, such as that which occurred on p50, in HEK293 cells. Interestingly for all genes tested, we demonstrated that the peroxynitrite/Tyr nitration pathway could be the primary inactivating mechanism (c-myc, ICAM-1, cyclin D1, cIAP-1, and IL-8) in both constitutively active (P19 and SH-SY5Y) and TNF-α-stimulated NF-κB (HEK293 and COS-1) systems.

Tyr nitration of p65 clearly triggered at least two important events: 1) replacement of p65/p50 with the repressive p50/p50 complex and 2) subsequent association of p65 with IκBα for export. IκBα is known to act as a shuttle between the nuclei and cytoplasm through a nuclear export signal (27, 28). Through its preferential interaction with IκBα, the nitrated p65 could be loaded on IκBα and subsequently exported from the nuclei. These two events could independently or synergistically inactivate p65 transcriptional activity.

These two events were further supported by the identification of specific Tyr nitration of p65 (Tyr-66 and Tyr-152). NF-κB translocation into nuclei is essential for its activation. On the contrary, S-nitrosylation at Cys-62 of the p50 subunit has been considered to be a major target of NO-triggered inhibition of NF-κB activity (18–20). NO can also stabilize IκBα in the cytoplasm by nitrosylating IκB kinase β at Cys-179 (21, 22, 29). Our study demonstrated an additional mechanism that directly and rapidly targeted the p65 subunit whose protein interaction and nuclear translocation property was altered by Tyr nitration. As a result, active NF-κB was rapidly inactivated. The nitration occurred at two specific Tyr residues of p65, Tyr-66 and Tyr-152, within its RHD (Fig. 6), which is known to be involved in interaction with other Rel family proteins and DNA binding (30). Tyr-66 is conserved in RHD-containing NF-κB family members, including p65, p50, p52, p100, p105, and c-Rel, but Tyr-152 is unique to p65. The anti-Ntyr antibody detected a weak band of p50 (Fig. 2), which could be due to nitration on the conserved Tyr-66 residue. In addition, nitration might cause a conformational change of RHD that disrupts interaction with p50 but enhances interaction with IκBα. However, this remains to be tested experimentally. NF-κB is known to activate inducible NO synthase to produce NO in response to stressful stimuli (31). Colasanti et al. (32) observed that exogenous NO suppressed inducible NO synthase mRNA expression and prevented TNF-α-stimulated NF-κB target gene expression. It was suggested that NO decreased NF-κB availability on the inducible NO synthase gene promoter. Our study provided an explanation for the feedback inhibition mechanism of NO by inactivating NF-κB through Tyr nitration of p65.

In most normal conditions, NF-κB exists in its inactive form by association with IκBα and is activated by a wide range of stimuli. Constitutively active NF-κB is widely known to be associated with various hematological malignancies and solid...
tumors including breast cancer, pancreatic carcinoma, and prostate cancer (1, 3, 33–38). In these cases, NF-κB induces survival cell, cell proliferation, angiogenesis, and metastasis of these cancers by promoting the expression of survival factors such as members of the cellular inhibitor of apoptosis family (cIAP-1, cIAP-2, and X-linked inhibitor of apoptosis protein), the Bcl-2 homologues (Bcl-2, Bfl-1/A1, and Bcl-XL), cyclin D1, and c-Myc (23, 39, 40). Our results suggest that the ability of NO-generating agents to rapidly and effectively inactivate NF-κB provides a potential route of drug intervention for various diseases, particularly cancers.

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