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Tyrosine Nitration of I κ B α : A Novel Mechanism for NF- κ B Activation

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Abstract

The NF- κ B family of transcription factors is an important component of stress-activated cytoprotective signal transduction pathways. Previous studies demonstrated that some activation mechanisms require phosphorylation, ubiquitination and degradation of the inhibitor protein, I κ B α . Herein, it is demonstrated that ionizing radiation in the therapeutic dose range stimulates NF- κ B activity by a mechanism in which I κ B α tyrosine-181 is nitrated as a consequence of constitutive NO[•] synthase activation, leading to dissociation of intact I κ B α from NF- κ B. This mechanism does not appear to require I κ B α kinase-dependent phosphorylation or proteolytic degradation of I κ B α . Tyrosine-181 is involved in several noncovalent interactions with the p50 subunit of NF- κ B stabilizing the I κ B α /NF- κ B complex. Evaluation of hydrophobic interactions of I κ B α /p50 based on the crystal structure of the complex is consistent with nitration disrupting these interactions, and dissociating the I κ B α /NF- κ B complex. Tyrosine nitration is not commonly studied in the context of signal transduction. However the present results indicate that tyrosine nitration is an important post-translational regulatory modification for NF- κ B activation and possibly for other signaling molecules modulated by mild and transient oxidative and nitrosative stresses.

The Rel/NF- κ B family of transcription factors mediate cellular responses to oxidative and other stresses (1). NF- κ B transcription factors are formed by the homo- or heterodimerization of proteins of the Rel family including p50, p52, p65 (RelA), c-Rel and RelB. The most abundant and best-understood dimer is p65/p50. This dimer exists in the cytoplasm complexed with an inhibitor protein, I κ B α , that masks the NF- κ B nuclear localization sequence (2). Stimulation of cells with cytokines such as tumor necrosis factor (TNF α) results in the phosphorylation, ubiquitination and degradation of I κ B α , permitting the unmasked p65/p50 heterodimer to translocate into the nucleus (2).

Ionizing radiation (IR) also activates NF- κ B by mechanisms not fully understood. At high IR doses (>10Gy) activation appears similar to that observed for TNF α . DNA damage-inducible

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kinases, ATM and DNA-PK, activate I κ B α kinases (e.g., IKK β) stimulating the phosphorylation, ubiquitination and proteasome degradation of I κ B α (1,3). IKK β activity and I κ B α degradation, however, do not appear necessary for NF- κ B activation at lower, therapeutic doses of IR, e.g. (4,5). Indeed, IR inhibits proteasomal activities at doses as low as 0.2 Gy with maximal inhibition at 2 Gy (4,6). IKK β has other functions including phosphorylation of S536 in the transactivation domain of the p65 subunit (7–10). Serine-536 phosphorylation is independent of I κ B α phosphorylation and important for enhancing p65 transactivation potential and modulating gene target selection.

Recent studies demonstrate that NO $^{\bullet}$ and a metabolic derivative, peroxynitrite (ONOO $^{-}$) modulate NF- κ B activity (11–15). For example, addition of a ONOO $^{-}$ donor to cultured cells stimulates NF- κ B reporter activity without I κ B α degradation (11). These findings and the evidence that a Ca $^{2+}$ dependent constitutive NO $^{\bullet}$ synthase activity in epithelial cells is transiently stimulated by low IR doses (16–19) prompted the following investigation of NO $^{\bullet}$ signaling in IR-induced activation of NF- κ B.

Materials and Methods

Cell Culture, Irradiation, and Transfection

CHO-K1 and MCF-7 cells were cultured and irradiated at a dose rate of 2 Gy/min with a ^{60}Co source as previously described (18). Cells were transfected with the LipofectAMINE PLUS $^{\text{TM}}$ kit (Invitrogen).

Reagents

Primary antibodies used: actin, nuclear lamin A/C, I κ B α , NOS1, IKK β and p65 (Santa Cruz Biotechnology); nitro-tyrosine (Upstate Biotechnology); p50, phospho-S32/36-I κ B α , and 9E10 epitope of c-Myc (Cell Signaling).

Wild type pCMV-I κ B α from Clontech has two ATG start sites with the second having an optimal Kozak sequence. For this reason an I κ B α doublet was detected after SDS polyacrylamide gel electrophoresis in early experimentation. Both proteins were, however, equally nitrated (e.g. Fig. 2A,E; 4A). Amino acid substitution mutants were constructed from pairs of point mutation primers by PCR technology with wild type pCMV-I κ B α as the initial template. Mutations were verified by full-length sequencing. Another set of mutants was prepared with an N-terminal c-Myc epitope tag to facilitate analysis.

The luciferase-based reporter construct of NF- κ B (pNF- κ B-luc) was also purchased from Clontech. Luciferase activity was measured in cell lysates 24 hr after IR exposure with a Luciferase Reporter Gene Assay Kit (Packard Bioscience) according to the manufacturer's directions.

The human shRNA IKK β plasmid was provided by Upstate Biotechnology. The dominant negative NOS-1 mutant (HemeRedF) and its effects on expression and activity of NOS-1 in CHO and other cells have been described (17,20,21). Mouse NOS-1 siRNAs and All Stars Negative Control siRNA were purchased from QIAGEN. HiPerFect Transfection Reagent (QIAGEN) was used for transfection of CHO-cells with siRNAs. Cells were seeded and transfected on the same day according to the manufacturer's reverse transfection protocol.

Measurement of Nuclear NF- κ B DNA Binding Activity

CHO-cells were seeded 48 h before radiation in 100-mm dishes and transfected the same day with the NOS1 siRNAs or 24 h later with a plasmid expressing the HemeRedF NOS-1 mutant. Incubation with 100nM L-NNA was performed 4 h before radiation. NF- κ B DNA binding

activity in nuclear extracts prepared as in (22) was measured with a NF- κ B p65 ELISA Kit (Stressgen Bioreagents) according to manufacturer's recommendations.

Biochemical Analyses

Immunoprecipitation and Western blotting methods have been described (17) (20). Protein detection was by chemiluminescence with alkaline phosphatase-conjugated secondary antibodies or with secondary antibodies conjugated with infrared fluorescent dyes and imaging with the Odyssey® Infrared Imaging System (Li-Cor® Biosciences). Cellular NOS activity was measured with an arginine to citrulline assay as previously described (17,20).

Mass Spectrometry

All proteins were resolved by one-dimensional SDS-PAGE and silver stained. After destaining, proteins were in-gel digested with modified porcine trypsin (0.6 μ g, Promega) alone or with *S. aureus* V8 protease (0.6 μ g, Sigma Chemical Co.) for 12 hrs according to the method of Shevchenko (23). The resultant peptides were purified with Poros 20 R2 reverse phase packing (Applied Biosystems) and subjected to direct infusion nanospray using NanoES spray capillaries (PROXEON, Odense, Denmark) on an Applied Biosystems QSTAR® pulsar XL mass spectrometer. MS spectra were collected in positive mode with an ion spray voltage of 800 volts. Subsequent MS/MS spectra were collected and amino acid sequences obtained using BioAnalyst software.

Structural Analysis

To determine the optimal geometry of nitro-tyrosine, we performed quantum mechanical calculations using density functional theory with 6-311+G(d,p) basis set, B3PW91 hybrid functionals, default spin configuration, and net molecular charge of zero (24). Self-consistent field was calculated directly with convergence limit of 2×10^{-5} . These parameters ensured that optimized structure of nitro-tyrosine was available for calculation of its various structural and chemical properties. The force constants for bonds, bond angles, and dihedrals were calculated using the Hessian matrix. Subsequently, charge distribution and electronic polarizability of nitro-tyrosine were calculated in water solution to fully characterize the properties of this modified amino acid. These properties were properly entered in the CHARMM22 (25) parameter and topology files for use in NAMD2, a molecular dynamics algorithm (26,27). The validity of quantum mechanical calculations was established by ^{13}C NMR spectroscopy using commercially available 3-nitro-tyrosine ethyl ester in D_2O (see supplemental data).

Two X-ray structures of I κ B α /NF- κ B complex are found in the Protein Data Bank – 1NFI (2.7 Å resolution) (28) and 1IKN (2.3 Å resolution) (29). The R-values for both structures were 0.223. The sequence enumerations are identical for I κ B α and p65 but are shifted by 3 residues for the p50 subunit. The 1IKN structure was used for our analysis. Energy minimization was performed for 2500 steps enabling energetic relaxation of the system.

For the HINT calculations, the PDB coordinates of control and nitrated I κ B α /NF- κ B complexes at the end of minimization were used. We defined Y181 or nitro-Y181 as structure “A” and the remainder of the I κ B α /NF- κ B complex as structure “B”. The HINT program was then used to evaluate a comprehensive set of non-bonded interactions between structures “A” and “B” (hydrogen bonding, acid-base, hydrophobic-hydrophobic, acid-acid, base-base, and hydrophobic-polar) (30–35).

Results

Inhibiting NOS-1 blocks IR-stimulated NF- κ B activity

Initial experiments tested whether radiation induced NOS-1 activity contributed to an increase of NF- κ B promoter activity measured with a luciferase based reporter assay. CHO cells were co-transfected with the reporter plasmid and either an empty vector or the dominant negative mutant of NOS-1 (HemeRedF) whose expression was previously shown to inhibit IR-activated NOS activity in CHO cells (17,20). A single IR exposure of 5 Gy stimulated a 1.5–3 fold increase in NF- κ B dependent luciferase activity measured at 24 h post-IR (e.g. Fig. 1A,B,G; 2G). This increase in activity was similar to what others have observed with diverse cell types after an exposure to IR (36–38). Expression of HemeRedF completely inhibited IR-stimulated reporter gene expression and reduced basal reporter activity by 40 to 50% (Fig. 1A). The effect of genetically inhibiting NOS-1 activity on NF- κ B promoter activity was confirmed pharmacologically with the NOS inhibitor, N^G-Nitro-L-arginine (L-NNA). Incubating cells with L-NNA 4 hr prior to radiation significantly reduced both basal and IR-induced NF- κ B promoter activity (Fig. 1B,G; 2G).

To further validate the role for NOS-1 in NF- κ B activation by IR, cells were transfected with control and siRNA directed against NOS-1. The inset of Figure 1C demonstrates that both siRNAs tested were efficient in knocking down expression of NOS-1 in CHO cells. We compared the relative effects of siRNA transfection, expression of the NOS-1 mutant, HemeRedF, and the chemical inhibitor, L-NNA, with respect to their relative abilities to inhibit basal and IR-induced NF- κ B. NF- κ B activity for these analyses was measured using nuclear extracts in an ELISA assay for p65 binding to a NF- κ B specific oligonucleotide consensus sequence. Results in Figure 1C show that all three methods of inhibiting NOS-1 activity were effective at blocking IR-stimulated NF- κ B activity although the molecular approaches appeared more effective. Further validation was obtained by following nuclear translocation of p65 subsequent to radiation. As shown in Fig. S3 of Supplemental Data, nuclear isolates were probed for p65 by Western blot analysis normalized with respect to nuclear lamin levels. Nuclear accumulation of p65 increased within 10 min of irradiation by a mechanism inhibited by L-NNA.

Activation of NF- κ B by a low IR dose does not stimulate I κ B α phosphorylation

We tested whether low IR doses activated IKK measured as I κ B α S32/36 phosphorylation and proteolysis. By this assay, IKK activity was not stimulated at the IR doses used (≤ 5 Gy) (Fig. 1D). Control experiments with TNF α stimulation showed enhanced, but transient I κ B α Ser32/36 phosphorylation and progressive decrease in I κ B α protein levels indicating that this IKK-dependent activation mechanism was intact. Incubation with L-NNA or expression of HemeRedF did not inhibit I κ B α S32/36 phosphorylation stimulated by TNF α (Fig. 1E,F).

NF- κ B activation by TNF κ or IR at 5 Gy were also compared in cells transfected with shRNA specific to IKK β to abrogate this activation pathway for NF- κ B (Fig. 1G). A co-transfected luciferase reporter construct was used to assess cellular NF- κ B activity. Cells expressing shRNA showed IKK β protein levels less than 10% of control cells, transfected with empty vector. This inhibition of IKK β expression with shRNA reduced NF- κ B basal activity by 50% and completely blocked stimulation by TNF α . In contrast to these observations with TNF α , IR-stimulated NF- κ B activity was only reduced by approximately 50% with IKK β shRNA expression. The remaining activity was inhibited by treatment with L-NNA (Fig. 1G). Since both basal and IR-stimulated NF- κ B activities were reduced by IKK β shRNA expression, the fold-activation with IR achieved in these cells was not significantly different from that of control, IKK β -expressing cells.

Radiation stimulates the tyrosine nitration of I κ B α

IR-activation of NOS stimulates ONOO⁻ generation detected as tyrosine nitration of a number of proteins (17). We tested whether IR at 5 Gy stimulated the nitration of I κ B α using CHO cells transfected with human wild type I κ B α . Anti-nitro-tyrosine immunoprecipitates from lysates of control and irradiated cells were analyzed by Western blot for I κ B α (Fig. 2A). IR stimulated oscillating changes in I κ B α tyrosine nitration with an initial maximum at 10–20 min post-IR and a second maximum at $\approx$ 40 min. Similar results were obtained with endogenous I κ B α in MCF-7 breast carcinoma cells (Fig. 2B). The reciprocal experiment with immunoprecipitation of I κ B α followed by blotting with anti-nitro-tyrosine IgG showed the same oscillations in tyrosine nitration of I κ B α without changes in I κ B α protein expression levels (Fig. 2C). Comparing amounts of endogenous tyrosine-nitrated I κ B α with total I κ B α suggests that up to 25% is transiently nitrated 15 min post-IR (Fig. 2D). Basal and IR-induced tyrosine nitration of I κ B α are both significantly inhibited by expression of HemeRedF or by incubating cells with L-NNA (Fig. 2E).

Previous studies demonstrated oscillations of NF- κ B DNA binding that correlated with oscillations in total I κ B α protein levels following TNF α treatment of cells (39,40). The present work with low doses of IR, in contrast, showed no measurable change in total cellular I κ B α levels after IR at 5 Gy (Fig. 2A,C,E). Thus, it is unlikely that selective proteolysis of nitrated I κ B α accounts for the observed oscillations in I κ B α nitration following an IR exposure. Proteolytic degradation of I κ B α is not a requirement for NF- κ B activation, e.g. (11,41). Furthermore, IR inhibits proteasome activities (4,6).

Radiation dose response analyses comparing cellular NOS activity, I κ B α tyrosine nitration and NF- κ B transcription reporter activity

Our previous studies (17,18) using a fluorescent dye to measure reactive oxygen/nitrogen species demonstrated a dose response which saturated at doses >6 Gy. These findings were confirmed by measuring as a function of IR dose cellular NOS activity directly with an arginine-citrulline conversion assay or indirectly by measuring tyrosine nitration of I κ B α (Fig. 2F). Both measures of NO^{*} activity progressively increased with IR dose and reached relative plateaus at doses greater than 6 Gy. A similar dose response curve was observed for NF- κ B reporter activity (Fig. 2G). At IR doses above 8 Gy, L-NNA was a less effective inhibitor of NF- κ B activation. At 16 Gy or higher, inhibition of NOS-1 activity with LNNA had no effect on IR-induced NF- κ B activity.

Tyrosines 181 and 305 of I κ B α are nitrated after irradiating intact cells

A genetic approach was also used to determine sites of nitration. Each tyrosine of I κ B α was individually mutated to phenylalanine. CHO cells were transfected with plasmids expressing all 8 myc-tagged Y-F mutants and the myc tagged wild type I κ B α . Tyrosine nitration of the mutants and wild type were compared as a function of time following an IR exposure of 5 Gy by immunoaffinity purification of the nitrated proteins followed by Western blot detection with anti-myc (Fig. 3). To facilitate comparisons between blots, each blot included one lane of wild type of myc-tagged I κ B α obtained from cell lysates nitrated with 50 μ M ONOO⁻ and subsequently immunopurified with anti-nitro-tyrosine conjugated agarose beads. All single mutants demonstrated with approximately identical frequencies oscillating levels of nitrated I κ B α following radiation. However, for the Y181F and Y305F single mutants, the amplitude in IR-induced nitration was significantly less than that observed for wild type and the other tyrosine mutants. A double mutant (Y181F+Y305F) was constructed to test whether these two tyrosines were exclusively nitrated following radiation. As shown in the bottom panels of Fig. 3, the double mutant was not nitrated after a 5 Gy radiation exposure.

Peroxynitrite treatment of the NF- κ B/I κ B α nitrates tyrosines 181 and 305 of I κ B α and dissociates the I κ B α /NF- κ B complex

Initial experiments using the different I κ B α mutants and treatment of cell lysates with ONOO⁻ also demonstrated a high degree of specificity in the ONOO⁻ induced nitration of I κ B α . As observed with radiation only mutant proteins for tyrosines 181 and 305 showed significantly reduced nitration following a single bolus addition of ONOO⁻. The double mutant for these two tyrosines was not nitrated at all (Fig. 4A,B).

We attempted to validate the genetic evidence for ONOO⁻ induced nitration of these two tyrosines by mass spectrometry. The cell lysate with overexpressed I κ B α was nitrated with ONOO⁻ and nitrated I κ B α isolated by precipitation with anti-nitro-tyrosine IgG, resolved by gel electrophoresis and processed for mass spectroscopy. After proteolysis, five I κ B α peptides were identified for coverage of 21% and this included 4 of the 8 tyrosines of I κ B α (Table S1 of supplemental data). The tryptic peptide containing tyrosine-181 is over 40 amino acids long and was not detected in the mass spectra. Attempts with different peptide cutting agents to obtain an identifiable peptide containing tyrosine-181 proved unsuccessful. However, a 20 amino acid peptide of I κ B α (aa295–314) was sequenced and identified in the un-nitrated form and also as a peptide with a mass consistent with tyrosine nitration (+45Da, supplemental data, Fig. S1, S2). Our initial attempts to sequence the only tyrosine in this peptide, Y305, to confirm its nitration, have not been successful. Additional mass spectroscopic analysis of the I κ B α 295–314 peptide indicated that C308 was modified by propionamide (an acrylamide adduct) indicating that C308 was not oxidized by the ONOO⁻ treatment. Methionine-91 in the amino acid 88–95 peptide was also not oxidized. Both findings support the conclusion that a bolus ONOO⁻ treatment is relatively specific in its effects on amino acid modification of I κ B α (42, 43).

We tested whether *in vitro* nitration with exogenous ONOO⁻ dissociated the I κ B α /NF- κ B complex. A single bolus addition of ONOO⁻ was used since the short half life of ONOO⁻ (<1 sec) enhances specificity in its reactions, e.g. (42,43). Cell lysates prepared under mild non-denaturing conditions were treated with 200 or 400 μ M ONOO⁻. Two consecutive immunoprecipitations were performed. Agarose-conjugated anti-p65 IgG was used to pull down the NF- κ B/I κ B α and free NF- κ B complexes. After centrifugation to remove these complexes, the resulting supernatants were incubated with agarose-conjugated anti-I κ B α IgG to pull down free I κ B α . Western blots of the immunoprecipitates were probed with antibodies against p65, I κ B α and nitro-tyrosine. With increasing concentrations of ONOO⁻, a decreasing amount of I κ B α associated with p65 with a corresponding increase in free tyrosine-nitrated I κ B α (Fig. 4C). Tyrosine-nitrated I κ B α did not co-immunoprecipitate with NF- κ B (lanes 5, 6). For long exposure times and at high [ONOO⁻] a broad smear of nitro-tyrosine staining was observed in p65 immunoprecipitates but with no distinct band for I κ B α (lane 3). Neither p50 nor p65 were nitrated under these conditions (data is not shown) and the p50/p65 dimer remained intact as shown by co-immunoprecipitation (Fig. 4D). These results suggest that tyrosine-nitration of I κ B α dissociates the NF- κ B/I κ B α complex releasing the p50/p65 dimer.

Cell lysates were prepared from cells transfected with myc-tagged wild type I κ B α and mutants (Y42F, Y181F and Y305F) and treated with 200 μ M ONOO⁻. NF- κ B immunoprecipitates obtained with anti-p65 were probed for p65 and myc-tagged I κ B α . ONOO⁻ treatment decreases the amount of I κ B α associated with p65 in wild type and all mutants tested except for the Y181F mutant (Fig. 4E). These results suggest that Y181 is critical to the stability of the NF- κ B/I κ B α complex following ONOO⁻ treatment.

CHO cells were co-transfected with the NF- κ B reporter gene and either myc-tagged wild type or the myc-tagged I κ B α Y $\rightarrow$ F mutants. As expected, over-expression of wild type or mutants inhibited basal NF- κ B reporter activity ($\approx$ 90%). However, a significant IR-induced activation

(~1.4) was still observed in cells expressing wild type or the Y42F and Y305F mutants (Fig. 4F). This is observed if promoter activity is expressed in terms of absolute values or as ratios of reporter activities of irradiated to control cells. In contrast, IR-stimulated NF- κ B reporter activity was completely blocked in cells expressing the Y181F mutant, demonstrating an important role for Y181 in the mechanism for IR-induced activation of NF- κ B. Basal NF- κ B activity of Y181F I κ B α -mutant expressing cells was moderately higher relative to the basal NF- κ B activity of cells overexpressing wild type or the other I κ B α mutants (Fig. 4F). This is consistent with a previous analysis of different I κ B α mutants showing that a Y181A mutant had a slightly lower affinity for p50/p65 (44). The variability in basal activity (e.g. Fig. 5A) probably reflects difference in expression levels.

Tyrosine-42 was also examined because previous reports indicated that its phosphorylation was important for oxidative activation of NF- κ B without degradation of I κ B α (41,45). However, mutation of Y42 to phenylalanine was without effect on either the stability of the NF- κ B/I κ B α complex after ONOO⁻ treatment (Fig. 4E) or IR-induced NF- κ B promoter activity (Fig. 4F).

Mutation of tyrosine-181 blocks IR-but not TNF α induced NF- κ B activity

The effects of overexpressing wild type I κ B α or the Y181F and S32A/S36A mutants of I κ B α on IR and TNF α stimulated NF- κ B activities were compared in MCF-7 cells. Serine-32 and -36 are phosphorylated by an IKK-dependent mechanism and thus the double mutant at these sites is a super-repressor for those activation mechanisms that proceed solely through IKK (1,39). MCF-7 cells demonstrate a relatively weak NF- κ B response to TNF α compared to other cell types reflecting the variable amount of TNF receptor in these cells (46). Nonetheless the results in Figure 5 and in Fig. 1G demonstrate that as with other cell types, the TNF α response proceeds through a mechanism involving IKK and serines-32 and 36. The experimental results are presented in absolute amounts of reporter luciferase activity (Fig. 5A) or as ratios of treated versus control activities (Fig. 5B). Expression of the S32A/S36A double mutant was significantly more effective than wild type I κ B α in suppressing TNF α -stimulated NF- κ B activity. The Y181F mutant and wild type I κ B α , in contrast, were no different in their effectiveness as inhibitors of TNF α -stimulated NF- κ B activity. Different results were obtained with IR as the activating mechanism. The Y181F mutant was significantly more effective at suppressing IR induced NF- κ B activity but was without any super-repressor activity with TNF α as the inducing agent. The results with the S32A/S36A mutant and IR parallel the findings provided above demonstrating that IR at the doses used here did not stimulate I κ B α phosphorylation and degradation and that blocking expression of IKK with shRNA only partially inhibited IR stimulated NF- κ B activation (Fig. 1G)

A structural analysis of the effects of Y181 nitration on the I κ B α /NF- κ B complex

The experimental results with IR and exogenous ONOO⁻ coupled with the site-directed mutagenesis studies strongly support a mechanism of Y181 nitration in NF- κ B activation by IR. Other oxidative modifications of I κ B α and nitration of either p50 or p65 after IR or ONOO⁻ treatments were not detected. To further substantiate a role for Y181 nitration in IR-induced dissociation of the I κ B α /NF- κ B complex an assessment of hydrophobic interactions of Y181 with neighboring residues was made and molecular dynamics simulations were performed.

A computational chemistry program HINT (Hydrophobic INteractions) permits the quantitative analysis of all possible noncovalent atom-atom interactions including hydrogen bonding, coulombic, acid-base, and hydrophobic using the crystal structure of proteins (47–49). HINT uses empirically derived constants based on thermodynamic hydrophobicity values from solvent partition measurements. The more positive the HINT value the more energetically

favorable the change in free energy. HINT calculations have accurately estimated changes in free energy resulting from site-specific mutations and their effect on hemoglobin dimer-tetramer assembly (48,49). Using HINT calculations, the interactions of Y181 or nitro-Y181 with the remainder of the NF- κ B/I κ B α complex were compared on an atom-by-atom basis with an 8 Å cutoff.

The initial calculations required a quantum-mechanical comparison of the unmodified tyrosine residue with the nitrated form to generate topology files for tyrosine and nitro-tyrosine (Fig. S4, Supplemental data). These calculations demonstrated an altered side-chain electron distribution that accounts for the measured decrease in pK of the phenolic group from ~10 to ~7 (50, 51). The portion of nitro-tyrosine containing the NO₂ group was highly electronegative with the most electropositive portion localized over the C $_{\alpha}$ and C $_{\beta}$ atoms. This results in a dipole moment vector oriented almost parallel with the C $_{\epsilon}$ -NO₂ bond (Fig. S4, Supplemental Data). The magnitude of the calculated dipole moment of nitro-tyrosine was 5.78 Debye, compared with 3.63 Debye for tyrosine. Without nitration, the tyrosine dipole moment vector was oriented along the long axis of the side chain aligning the hydroxyl group of the phenol ring with C $_{\beta}$ and C $_{\alpha}$ atoms.

Crystallographic studies of the NF- κ B/I κ B α complex show that I κ B α is orientated such that fingers 3/4, 4/5, and 5/6 of I κ B α contact the p50 subunit (28,29). Y181 and N182 extending from finger 3/4 have multiple contacts with p50. Y181, in particular, has an important role in these interactions since it forms hydrogen bonds with p50 K252 and R258, π -stacks with Y351, and makes multiple van der Waals contacts with A260, P327, and L349. Table 1 shows results on an atom-by-atom basis of HINT calculations using a cut-off distance between atoms of 8 Å. 'Nitration' of Y181 using the above generated topology file cause significant destabilizing changes in these interactions as seen by the net negative increase in total HINT score. A more instructive presentation of the HINT scores is shown in the Summary inset to Table 1 where the total HINT scores for I κ B α Y181 interactions with p50 and with other residues of I κ B α are separated. Nitration results in a significant destabilization of p50-I κ B α interactions as indicated by a net negative increase in HINT score from -218 to -1970. Assuming approximately 1 kcal/mole per 500 HINT, this represents a change in free energy of approximately 3 kcal/mol. In contrast, the 'nitration' induced change in HINT scores for Y181 interactions with other I κ B α residues within 8 Å is consistent with a net stabilizing effect. Major contributions to this positive interaction are the acid/base and hydrogen bond interactions of the nitro-group with R140 of I κ B α (Table 1, Fig. 6A).

To experimentally test for this, the R140A I κ B α mutant was constructed, and the relative affinities of wild type and R140A myc-tagged I κ B α to NF- κ B compared by co-immunoprecipitation with anti-p65 antibody (Fig. 6B). The affinity of the R140A mutant for NF- κ B is considerably reduced relative to wild type even in the absence of nitration. These results do not allow for any statement on the role of this particular interaction on the effect nitration on I κ B α /NF- κ B stability. However, they underline the importance of this surface area of I κ B α in its interactions with p50/p65 and the potential for their disruption by tyrosine nitration.

Discussion

The above experimentation indicates a new mechanism for NF- κ B activation. IR is shown to stimulate NF- κ B activity by a mechanism in which I κ B α Y181 is nitrated as a consequence of NOS-1 activation, leading to dissociation of intact I κ B α from NF- κ B. Hence, this mechanism of NF- κ B activation does not depend on IKK dependent phosphorylation and proteolytic degradation of I κ B α . Modeling of free energy changes is consistent with the experimental findings that IR-induced Y181 nitration disrupts the noncovalent interactions of Y181 with

p50 dissociating the I κ B α /NF- κ B complex. The apparent lack of I κ B α degradation following treatment of cells with low IR doses is also in accord with findings that IR at doses > 0.2 Gy significantly inhibits proteasome activities (4,6). The IR dose response analysis demonstrates a progressive increase in NF- κ B activation up to the highest dose tested, 32 Gy. However, only at IR doses below 8 Gy is substantial inhibition observed with the NOS inhibitor L-NNA. Similar dose responses are observed for IR-induced I κ B α nitration and NOS-1 activation.

The experimental results suggest that NF- κ B is activated by IR through both, IKK β -independent and IKK β -dependent mechanisms. The IKK α -independent pathway involving tyrosine nitration of I κ B α is prominent at IR doses $\leq$ 8 Gy whereas IKK α -dependent pathway involving I κ B α phosphorylation/proteolysis becomes more prominent at IR doses > 8 Gy. Dose dependent mechanisms are also indicated in the IKK- β knockdown experiments. Whereas shRNA treatment completely abrogates TNF α -induced NF- κ B activity, it only partially decreases IR-induced activity. The remaining activity is inhibited by NOS inhibitor L-NNA.

Analysis of the kinetics of NF- κ B activation following low or high dose IR is also indicative of different mechanisms. A previous study using electrophoretic mobility shift analysis monitored activation of NF- κ B in HeLa cells after 20 Gy (52). No activation was observed at 30 min, a slight increase at one hour and maximal activation at two hours. This contrasts with the responses obtained within minutes of exposure to lower doses of IR (Fig 1C,2) or to TNF α (39). IR-stimulated nitration of I κ B α is observed at 5 min post-irradiation at 5 Gy, the earliest time point tested (Figure 2). In contrast I κ B α Ser-32/36 phosphorylation is first observed at 45 min post-irradiation with 20 Gy (39).

The IKK β dependent and independent pathways are not mutually exclusive. This is suggested by the partial inhibition of NF- κ B signaling at the lower doses of IR by shRNA knockdown of IKK β . However, the IKK β -mechanisms may differ depending on IR dose. For example, different mechanisms may reflect the cellular localization of the oxidative/nitrosative events: DNA damage in the nucleus and oxidative/nitrosative events in the cytoplasm. Further experiments are necessary to test this hypothesis. IKK β can also modulate NF- κ B transcriptional activity in the cytoplasm through an I κ B α -independent mechanism: phosphorylation of the p65 subunit in its transactivation domain at S536 (7–10).

NF- κ B activation by reactive nitrogen species has been previously investigated using a ONOO⁻ donor, SIN-1 (11). NF- κ B activity measured with a luciferase-based reporter assay was stimulated by a mechanism not blocked by the proteasome inhibitor, MG132, nor was I κ B α degradation associated with the stimulation. These experimental findings directly relate to the results reported here with IR. Thus, our results provide a mechanism for how SIN-1 and other cellular ONOO⁻ generating processes can activate NF- κ B signaling.

A previous analysis of several I κ B α mutants demonstrated that the Y181A mutation was most defective for complex formation with NF- κ B (44). However, all I κ B α mutants examined including Y181A still bound NF- κ B with nanomolar affinities suggesting that several binding elements contribute to the overall stability of the NF- κ B/I κ B α complex. This conclusion would appear to be in conflict with the present findings that mutation of Y181 alone completely blocks IR stimulated NF- κ B DNA binding activity and ONOO⁻ induced disruption of the NF- κ B/I κ B α complex. A likely explanation is that nitration of tyrosine is a much more disruptive protein modification than substitution of an alanine for tyrosine. Besides introducing a bulky substituent, tyrosine nitration significantly alters the dipole moment of tyrosine and reduces the phenolic pK by 2–3 units effectively introducing a net negative charge in a relatively non-polar restricted space. Other studies have demonstrated that nitration of a specific tyrosine in proteins can have significant structural and functional consequences for proteins, e.g. (53–57). It is also important to note that the studies by Huxford *et al* (44) used a bacterial expression

system with a truncated I κ B α lacking both N- and C-terminal elements, including Y305. Nitration of Y305 may have an important role in modulating the stability of the NF- κ B/I κ B α complex.

Tyrosine-305 of I κ B α is also nitrated following radiation or an acute ONOO⁻ treatment. We were unable to model the effects of Y305 nitration on the I κ B α /NF- κ B complex stability because both available crystal structures of I κ B α /NF- κ B lack the C-terminal regions containing Y305. From the 1IKN structure, it is clear that a turn in secondary structure occurs around P215 and the C-terminal portion of I κ B α wraps on itself and runs along the interface with p65 and p50 subunits of NF- κ B. Tyrosine-305 is part of this C-terminal sequence and its nitration can presumably contribute to disruption of non-covalent interactions stabilizing the I κ B α /NF- κ B complex. Is Y181 and Y305 nitration cooperative and is nitration of both residues required for rapid dissociation of the complex? From results of our work, it appears that nitration of Y181 is alone sufficient to destabilize the signaling complex, but the kinetics of dissociation of I κ B α from NF- κ B may be affected by additional nitration of Y305. A previous report also provides evidence that phosphorylation of Y305 increased the *in vivo* stability of I κ B α (58). It is possible that its nitration serves a similar purpose. Some investigations have suggested that nitro-tyrosine may mimic phospho-tyrosine binding sites, e.g. (59). Further experimentation will be necessary to test these mechanisms involving Y305.

Experiments described here and in other published work demonstrate a high degree of selectivity in what tyrosines are nitrated by an acute ONOO⁻ treatment (60). If complexed with p50/p65, I κ B α is nitrated on only 2 of 8 tyrosines following an IR exposure of intact cells or treatment of cell lysates with ONOO⁻. With the same *in vitro* conditions attempts to nitrate purified I κ B α with ONOO⁻ have proven unsuccessful (C.S. Rabender, unpublished data). This suggests that the tertiary or quaternary structures of I κ B α are important in determining susceptibility to nitration by ONOO⁻. An analysis of nitrated proteins suggests that no specific structural feature alone determines nitration (60,61). Tyrosines located in loop structures have increased probability for nitration (60,61) and Y181 is located in the β -turn loop between the 3rd and 4th ankyrin repeats.

No amino acid consensus sequence has been identified although it has been speculated that nitrated tyrosines are generally but not always near acidic amino acid residues (60). This is true for Y305 with one glutamate in close proximity (KPFLY³⁰⁵EIK), but this is not the case for Y181 (KATNY¹⁸¹NGHT). There are also no acidic amino acids from p50 or p65 at the interface near Y181 of I κ B α that can contribute to this possible electrostatic environment. A more recent report on endogenously nitrated proteins of the brain argues for the importance of a positively charged amino acid near sites of nitration (61). This criterion is fulfilled by both Y181 and Y305 of I κ B α .

The short half-life of ONOO⁻ predicts that proximity of the protein to ONOO⁻ source is also important in selectivity (19,60). The primary source of IR-stimulated cellular ONOO⁻ is the mitochondrion either by activity of a mitochondrial NOS-1 isoform or as a consequence of respiration generated superoxide anion that reacts with relatively stable NO[•] produced elsewhere in the cell (16,19,60,62). A reversible mitochondrial protein tyrosine nitration initiated by hypoxia/reoxygenation has been described (62) and there are reports of mitochondrial localization of I κ B α /NF- κ B (63,64).

Transient and localized generation of ONOO⁻ may also explain why the results presented here differ from those obtained in some but not all studies on the effects of reactive nitrogen species on NF- κ B activity (13–15,65). High concentrations of NO[•]/ONOO⁻ donors and/or prolonged exposure times inhibit NF- κ B activity, e.g. 6–24 hr (15). This contrasts with the transient generation of ONOO⁻ achieved by a short IR exposure of cells or a single bolus addition of

ONOO⁻ (half life <1 sec) to cell lysates. The transient nature of these treatments would be predictive of a higher degree of specificity in the nitration process. This is not only seen in terms of what tyrosines are nitrated but also in terms of whether other ONOO⁻ induced oxidative events have occurred. Under the conditions used in the present experiments there is no evidence for nitration of either p65 or p50. There is also no evidence for the oxidation of either a cysteine or methionine. On the other hand a single Y181F mutation was sufficient to block IR stimulated NF-κB DNA binding activity and ONOO⁻ induced dissociation of the NF-κB/IκBα complex. Combined with the effects of NOS inhibition and structural analysis, this is compelling evidence for a key role for Y181 nitration in IR stimulation of NF-κB activation.

An important characteristic of signal transduction pathways is their reversibility. The transient and relatively mild oxidative treatments used here may also have facilitated the apparent reversibility of the IR-stimulated IκBα tyrosine nitration. Although the mechanism of reversibility is not known, proteolytic degradation followed by resynthesis does not appear to be involved. This also is the case with the reversible nitration of mitochondrial proteins after hypoxia-reoxygenation (62). There are reports of denitrase activities in eukaryotic cells but underlying denitration mechanisms of eukaryotic cells remain undefined (66–68).

Because tyrosine-nitration is not commonly studied in the context of signal transduction, it may be an undiscovered but important component for NF-κB activation by stimuli other than IR. A previous report that mitochondria-generated superoxide anion and ONOO⁻ are important for TNFα-stimulated NF-κB activation is interesting in this regard (69). Nitration and disruption of the interactions between Y181 of IκBα with p50 may be representative of a post-translational modification and structural motif critical for the stability of other protein complexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

NOS-1, constitutive NO[•] synthase; TNF, tumor necrosis factor; IKK, IκB kinase; IR, ionizing radiation; L-NNA, N^G-Nitro-L-arginine.

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176x240mm (300 x 300 DPI)

Figure 1.

IR and TNF α activation of NF- κ B. **A.** CHO-cells were co-transfected with pNF- κ B-luc and either pHemeRedF (dominant negative mutant of NOS-1) or an empty vector as a control. Cells were radiated (5 Gy) 48 h after transfection and luciferase activity was measured in cell lysates 24 h later. **B.** 44 hrs after transfection with pNF- κ B-luc, cells were treated with 100 nM L-NNA for 4 h prior to a 5 Gy IR exposure. Experimental data (for A and B) are presented as means $\pm$ SD for quadruplicate samples and are representative of experiments performed in triplicate. **C.** CHO-cells were seeded and transfected with NOS1 siRNAs on the same day or with HemeRedF plasmid 24 hours later. Incubation with 100nM L-NNA was performed 4 h before radiation. Cells were radiated (5 Gy) 48 h after seeding and harvested at the given time-

points after IR. Nuclear extracts of the cells were prepared and normalized relative to the total protein concentration. The ELISA assay was performed to measure specific DNA binding activity of NF- κ B in the nuclear extracts. Experimental data are presented as means $\pm$ SD for triplicate samples. Embedded panel shows level of NOS1 48-h after isRNAs transfection. **D.** MCF-7 cells were irradiated at 5 Gy and cell lysates analyzed by immunoblotting for phospho-S32/36-I κ B α (red) and I κ B α (green). As a positive control cells were treated with 10 nM TNF α . Equal loading was verified by blotting with anti-actin (bottom panel). **E.** Cells were pretreated with 100 nM L-NNA for 4 h prior to adding 10nM of TNF α . Equal loading was verified by western blotting of cell lysates with anti-actin (bottom panel). **F.** MCF-7 cells were transfected with pHemeRedF or empty vector as a control. Cells were treated with TNF α (10nM) 48 h after transfection. Equal loading was verified by immunoblotting cell lysates with anti-actin (bottom panel). **G.** MCF-7 cells were co-transfected with pNF- κ B-luc and either pIKK- β shRNA or the empty vector as a control. Cells were radiated (5 Gy) or treated with TNF α 48 h after transfection and luciferase activity was measured in cell lysates 24 h later. L-NNA (100nM) was added to the cell cultures 4 hrs prior to IR exposure or TNF α treatment. Experimental data are presented as means $\pm$ SD for triplicate samples. Right panel: Western blots of cell lysates validating the effectiveness of IKK β shRNA treatment – 48 h after transfection. Cell lysates were probed with anti-IKK β (green) and anti-actin antibody (red) as a loading control.

177x230mm (300 x 300 DPI)

Figure 2.

IR dose dependence of NOS-1 activity, NF- κ B induction and I κ B α tyrosine-nitration. **A.** Tyrosine nitration of I κ B α after IR (5 Gy) was monitored in CHO-cells transfected with pCMV-I κ B α -wild type and irradiated 48 h after transfection. Anti-nitro-tyrosine immunoprecipitates analyzed for I κ B α by Western blotting. Equal loading was verified by immunoblotting cell lysates with anti-I κ B α antibody (bottom panel). **B.** Radiation-stimulated tyrosine nitration of endogenous I κ B α in MCF-7 cells. MCF-7 cells were irradiated (5 Gy) and cell lysate prepared at the indicated time points. Anti-nitro-tyrosine immunoprecipitates were analyzed with anti-I κ B α antibody. For loading controls, equal amounts of each cell lysate were fractionated by electrophoresis and immunoblotted with anti-actin antibody. **C.** MCF-7 cells were irradiated as

in Fig. 2B. Anti-I κ B α immunoprecipitates were probed with anti-nitro-tyrosine antibody. For loading controls, equal amounts of each immunoprecipitate were fractionated by electrophoresis and blotted with anti-I κ B α . **D.** Whole-cell extracts from non-transfected cells were prepared at 0 (no IR), and 5 and 15 min post-IR (5 Gy). Anti-nitro-tyrosine immunoprecipitates from total cell extracts were analyzed by immunoblotting with anti-I κ B α and compared with 2% of total cellular I κ B α (lane 4). Cell lysates were probed with anti-actin antibody as loading control (bottom panel). **E.** NOS activity modulates I κ B α tyrosine-nitration after IR (5 Gy). CHO cells were co-transfected with pI κ B α and pHemeRedF or an empty vector. Alternatively, cells transfected with pI κ B α were treated with 100nM L-NNA 4 h prior to irradiation. The experimental protocol in Fig. 2C was used to determine the effects of inhibiting NOS activity by HemeRedF expression or incubating cells with L-NNA (upper panel). Bottom panels are loading controls of cell lysates probed with anti-I κ B α antibody. **F.** MCF-7 cells were lysed 15 minutes after exposure to different IR doses. Anti-nitro-tyrosine immunoprecipitates were analyzed by immunoblotting with anti-I κ B α . Cell lysates were probed with anti-I κ B α to verify equal loading (lower panel); Graph: Arginine-citrulline conversion assay with different doses of IR was performed as previously described (17,20). Results are presented as the average of triplicate samples $\pm$ SD; **G.** L-NNA inhibition of NF- κ B activity after different doses of IR. MCF-7 cells were transfected with pNF- κ B-luc and irradiated 48 h after transfection. Luciferase activity was measured in cell lysates 24 h later. Cells were incubated with 100 nM L-NNA for 4 h prior to a IR exposure.

177x133mm (300 x 300 DPI)

Figure 3.

Site-directed mutagenesis and identification of $\text{I}\kappa\text{B}\alpha$ tyrosines nitrated after radiation. CHO cells were transfected with cMyc-tagged $\text{I}\kappa\text{B}\alpha$ wild type, different Y $\rightarrow$ F mutants, and double mutant 181/305YY $\rightarrow$ FF and irradiated with 5 Gy 48 h after transfection. Cells were lysed at the certain time-points after IR. Anti-nitro-tyrosine immunoprecipitates were analyzed by immunoblotting with anti-cMyc (left panels). Cell lysates were probed with anti-cMyc to verify equal loading of cMyc-tagged $\text{I}\kappa\text{B}\alpha$ (right panels). As a positive control and to facilitate comparisons, cMyc-tagged $\text{I}\kappa\text{B}\alpha$ in cell lysates of over-expressing cells was nitrated with 50 μM ONOO^- . The nitrated $\text{I}\kappa\text{B}\alpha$ as immunopurified as described and run on each blot (the last line of the each panel).

177x204mm (300 x 300 DPI)

Figure 4.

Tyrosine-nitration of IκBα dissociates the IκBα/NF-κB complex. **A.** Cell lysates from CHO cells expressing IκBα wild type and different Y→F mutants were treated with ONOO⁻. Anti-nitro-tyrosine immunoprecipitates were analyzed by immunoblotting with anti-IκBα. Cell lysates were probed with anti-IκBα to verify equal loading (lower panel). Control – untransfected sample. **B.** Cell lysates from CHO cells expressing cMyc-tagged IκBα wild type and double mutant 181/305YY→FF were treated with ONOO⁻. Anti-nitro-tyrosine immunoprecipitates were analyzed by immunoblotting with anti-cMyc. Cell lysates were probed with anti-cMyc to verify equal loading of cMyc-tagged IκBα (lower panel). **C.** p65 and IκBα immunoprecipitates were obtained from ONOO⁻ treated cell lysates as described in the

text. Preliminary control experiments demonstrated that the amount of antibody used was sufficient to fully immunoprecipitate the target antigen. Samples from first (lanes 1, 2, 3) and second (lanes 4, 5, 6) immunoprecipitations were analyzed for p65 (upper panel) and I κ B α (middle panel) by immunoblotting. The blots were probed simultaneously with anti-nitro-tyrosine antibody (bottom panel). **D.** After ONOO⁻ treatment, anti-p65 immunoprecipitates were analyzed for p50 and I κ B α . The loading control was p65 (upper panel). **E.** CHO-cells were transfected with plasmids encoding c-myc-tagged wild type and the Y $\rightarrow$ F mutants of I κ B α . Cell lysates prepared 24 hours after transfection were treated with 200 μ M ONOO⁻. Anti-p65 immunoprecipitates were analyzed by Western blot for p65 and c-myc. The fluorogram from one of two experiments is shown. The fluorescence intensity readings from two different experiments were used to calculate the relative nitration-induced decrease of I κ B α associated with p65 calculated as average intensity of I κ B α normalized to the loading control (p65 intensity) of $\pm$ standard deviation. * – The p values were determined by Student's t-test relative to wild type transfected cells. **F.** DNA-binding activity of NF- κ B was estimated as in Fig. 1 after co-transfection of pNF- κ B-luc with I κ B α wild type and the indicated Y $\rightarrow$ F mutants. The increase in activity after IR was normalized relative to cells co-transfected with pNF- κ B-luc and empty vector. Data are means $\pm$ SD for quadruplicate samples and representative for experiments performed in triplicate. * – The p values were determined by Student's t-test relative to the wild type transfected cells.

82x120mm (600 x 600 DPI)

Figure 5.

I κ B α Y181F can play role of super-mutant in the radiation-dependent NF κ B activation. **A.** MCF-7 cells were co-transfected by pNF- κ B-luc with I κ B α wild type, or I κ B α Y181F mutant, or I κ B α S32A/S36A mutant. Cells were radiated (5 Gy) or incubated with TNF- α (10nM) 48 h after transfection and luciferase activity was measured in cell lysates 24 h later. **B.** Absolute luminescence values are provided in Fig.5A converted into reporter activity ratios of treated versus non-treated cells. Data are means $\pm$ SD for quadruplicate samples and representative for experiments performed in triplicate. *, ** – The p values were determined by Student's t-test relative to the I κ B α wild type transfected cells for radiated/non-radiated ratios. ** – The p values

were determined by Student's t-test relative to the I κ B α wild type transfected cells for TNF- α -treated/non-treated ratios.

82x114mm (600 x 600 DPI)

Figure 6.

The spatial relationship between NO₂ group of Y181 and R140 of IκBα. **A.** R140, an inherently electropositive residue, buffers the electronegative charge of nitrated Y181 via long-range electrostatic interactions as evidenced by out-of-plane rotation of the NO₂ group. Optimized structure of nitrated tyrosine indicates that the most optimal structure is when the NO₂ group is in the plane of the phenol ring. **B.** R140 of IκBα is responsible for stability of NF-κB/IκBα complex. CHO cells were transfected by wild type and R140A mutant myc-IκBα. Cell lysates were prepared 24 hours after transfection and immunoprecipitated by anti-p65 antibodies. Immunoprecipitates were analyzed by blotting with anti-p65 and anti-IκBα antibodies. Equal amount of cell lysates were used as a transfection control (bottom panel).

Table 1
HINT score calculations (Y181 before and after nitration with p50 and IκBα).

Before Nitration					After Nitration						
	Residue	Residue Atom	nitroTyr Atom	Interaction Score	Interaction Type	Residue	Residue Atom	nitroTyr Atom	Interaction Score	Interaction Type	
NF-κB (p50)	ARG258	NH2	CA	-15	Hydroph./Polar	PRO327	CD	OH	-23	Hydroph./Polar	
	ARG258	NH2	CB	-54	Hydroph./Polar	PRO327	CD	ON1	-16	Hydroph./Polar	
	ALA260	CB	CA	29	Hydrophobic	PRO327	CD	ON2	-50	Hydroph./Polar	
	ALA260	CB	CB	34	Hydrophobic	PRO327	CB	OH	-33	Hydroph./Polar	
	ALA260	CB	CD2	12	Hydrophobic	PRO327	CB	NE2	31	Hydrophobic	
	PRO327	CD	OH	-23	Hydroph./Polar	PRO327	CB	ON1	-77	Hydroph./Polar	
	PRO327	CB	OH	-34	Hydroph./Polar	PRO327	CB	ON2	-106	Hydroph./Polar	
	PRO327	CG	OH	-122	Hydroph./Polar	PRO327	CG	OH	-119	Hydroph./Polar	
	LEU349	CD1	OH	-49	Hydroph./Polar	PRO327	CG	NE2	57	Hydrophobic	
	LEU349	CD1	CD2	13	Hydrophobic	PRO327	CG	ON1	-93	Hydroph./Polar	
	LEU349	CD1	CE2	19	Hydrophobic	PRO327	CG	ON2	-424	Hydroph./Polar	
	TYR351	CB	OH	-45	Hydroph./Polar	LEU349	CB	ON1	-33	Hydroph./Polar	
	PRO352	CD	OH	-17	Hydroph./Polar	LEU349	CG	ON1	-15	Hydroph./Polar	
	ARG140	CB	OH	-14	Hydroph./Polar	LEU349	CD1	OH	-47	Hydroph./Polar	
IκBα	ARG140	NH1	OH	-15	Acid/Acid	LEU349	CD1	CD2	14	Hydrophobic	
	ARG140	NH1	CD2	13	Acid/Base	LEU349	CD1	NE2	142	Hydrophobic	
	ARG140	NH1	CE2	21	Acid/Base	LEU349	CD1	ON1	-853	Hydroph./Polar	
	ASP141	O	OH	-15	Base/Base	LEU349	CD1	ON2	-158	Hydroph./Polar	
	PHE142	O	CD1	-15	Hydroph./Polar	LEU349	CD2	NE2	23	Hydrophobic	
	PHE142	O	CE1	-15	Hydroph./Polar	LEU349	CD2	ON1	-100	Hydroph./Polar	
	ARG143	O	CA	-15	Hydroph./Polar	ARG140	NH1	NE2	238	Hydrogen Bond	
	ARG143	O	CB	-28	Hydroph./Polar	ARG140	NH1	ON1	995	Hydrogen Bond	
	ARG143	O	CD1	-14	Hydroph./Polar	ARG140	NH1	ON2	261	Hydrogen Bond	
	GLY144	CA	CE1	13	Hydrophobic	ARG140	NH2	NE2	16	Acid/Base	
	GLY144	CA	OH	-44	Hydroph./Polar	ARG140	NH2	ON1	113	Acid/Base	
	GLY144	CA	CD2	11	Hydrophobic	ARG140	NH2	ON2	31	Acid/Base	
	GLY144	CA	CE2	15	Hydrophobic	ASP141	O	OH	-12	Base/Base	
	ASN180	OD1	CA	-20	Hydroph./Polar	PHE142	O	CD1	-15	Hydroph./Polar	
	ASN180	O	CA	-82	Hydroph./Polar	PHE142	O	CE1	-15	Hydroph./Polar	
	ASN180	O	CB	-19	Hydroph./Polar	ARG143	O	CA	-15	Hydroph./Polar	
	ASN182	NI	CA	-350	Hydroph./Polar	ARG143	O	CB	-28	Hydroph./Polar	
	ASN182	NI	CB	-69	Hydroph./Polar	ARG143	O	CD1	-14	Hydroph./Polar	
	ASN182	OD1	CA	-12	Hydroph./Polar	GLY144	CA	CE1	13	Hydrophobic	
	ASN182	OD1	CB	-14	Hydroph./Polar	GLY144	CA	OH	-41	Hydroph./Polar	
	GLY183	N	CA	-13	Hydroph./Polar	GLY144	CA	CD2	12	Hydrophobic	
Total HINT Score					-904.132	GLY144	CA	NE2	32	Hydrophobic	
						GLY144	CA	ON1	-40	Hydroph./Polar	
						GLY144	CA	ON2	-34	Hydroph./Polar	
						GLY144	O	NE2	-33	Base/Base	
						GLY144	O	ON1	-76	Base/Base	
						GLY144	O	ON2	-48	Base/Base	
						THR179	O	NE2	-21	Base/Base	
						THR179	O	ON1	-157	Base/Base	
						THR179	O	ON2	-20	Base/Base	
						ASN182	NI	CA	-350	Hydroph./Polar	
						ASN182	NI	CB	-69	Hydroph./Polar	
						Total HINT Score					-1783

Summary of HINT Scores - IκBα Y181 with:		
	Before Nitration	After Nitration
p50	-218	-1970
IκBα	-686	+187

177x126mm (400 x 400 DPI)