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Original Research Communication

Peroxynitrite Induces an Alternative NF-κB Activation Pathway in L8 Rat Myoblasts

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ABSTRACT

The role of peroxynitrite in NF-κB activation remains controversial. This study investigated NF-κB activation by peroxynitrite in skeletal myocytes. Myocytes were treated with NO and peroxynitrite donors. Both NO and peroxynitrite caused NF-κB activation (measured by p65 nuclear translocation and luciferase expression). NO donor-induced NF-κB activation was transient, dependent on I-κB α degradation, and was decreased in the presence of I-κB α super-repressor. Conversely, peroxynitrite donors induced NF-κB activation that was dependent on tyrosine nitration of I-κB α, but independent of its serine phosphorylation and degradation. This activation did not decrease in the presence of I-κB α super-repressor. Prolonged exposure to peroxynitrite resulted in nontransient NF-κB activation and high iNOS expression. Proteasome inhibitor MG-132 did not diminish SIN-1-induced NF-κB activation. Tyrosine nitration inhibitor EGCG re-established transient NF-κB activation with I-κB α degradation after SIN-1 treatment. EGCG, but not MG-132 decreased SIN-1-dependent iNOS expression. Peroxynitrite activates NF-κB in skeletal myocytes through an alternative mechanism, in which I-κB α is nitrated on tyrosine and dissociated from NF-κB, thus enabling its nontransient activation. This resulted in prolonged iNOS expression. Hence, peroxynitrite may exacerbate inflammatory responses mediated by NF-κB. *Antioxid. Redox Signal.* 8, 639–652.

INTRODUCTION

Reactive nitrogen species (RNS) are highly active compounds that can cause cellular damage through oxidation and nitration of biomolecules. RNS include NO and its derivative peroxynitrite (ONOO). Peroxynitrite is produced under inflammatory conditions and has been implicated in promoting the development of various pathologies (28, 31). Nitrated tyrosine residues represent biological markers for peroxynitrite-induced tissue damage and have been detected in inflamed (10) and in aged (4, 11, 55) tissues. The role of RNS in the activation of the pro-inflammatory transcription factor NF-κB remains controversial. Several studies have demonstrated RNS-dependent activation of NF-κB (23, 35), whereas other studies have contradicted this (25, 36). Extracellular stimuli (6, 22, 23, 26, 27, 39, 43, 51) lead to the activation of I-κB kinase (IKK) that phosphorylates I-κB on two conserved serines (Ser32 and Ser36 in I-κB α). This phosphorylation marks I-κB for proteasomal degradation, resulting in the nuclear translocation and activation of NF-κB (24). This is considered the classic pathway of NF-κB activation. Several alternative pathways of NF-κB activation have been described. For example, hypoxia-reoxygenation has been shown to activate NF-κB through tyrosine phosphorylation of I-κB α (40).

Degenerative conditions that may develop in skeletal muscle include cachexia of cancer (50), inherited muscular dystrophies (45), loss of muscle mass that is age-associated or a consequence of prolonged limb immobilization (7, 34). The common denominators in all these wasting conditions are the induction of local inflammation, free radical production (17, 18), and the excessive proteolysis of muscle proteins (2, 15).

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In hindlimb unloading of young rats, an alternative NF-κB complex, which consisted of p50 homodimer bound to Bcl-3, was activated in the first days of muscle breakdown (20, 21). Similar indication for the activation of more than one type of NF-κB has also been reported in hindlimb immobilization of old rats (3). However, little is known about the role of RNS and NF-κB in skeletal muscle pathology. Therefore, in this study we attempted to investigate the molecular mechanisms of the activation of NF-κB by RNS in skeletal myocytes.

MATERIALS AND METHODS

Cell culture

The rat myoblast L8 cell line was a generous gift from Dr. E. Bengal from the Department of Biochemistry, Rappaport Faculty of Medicine, Technion, Israel. The cells were grown in Dulbecco's modified Eagle's medium (Biological Industries Inc., Kibbutz Bet Haemek, Israel) supplemented with 10% (v/v) Fetal Bovine Serum. The differentiation of the cells into mature myocytes was not required for this type of experiments, since previous studies demonstrated that NF-κB-dependent responses in myoblasts were similar to those of mature myocytes (30, 58).

Pharmacological treatment of cells

At the beginning of each experiment, culture medium was replaced by fresh medium in which various substances and reagents were dissolved. The following substances were used: (i) NO donor 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA-NONOate) (32), NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) (14), and peroxynitrite donor 3-morpholine-sydnonimine (SIN-1) (33) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Authentic peroxynitrite was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Stock solutions of 1 m*M* of the chemicals were prepared in 1 *M* NaOH, since RNS donors are stable in alkaline pH (29). RNS donors were added to the growth medium at final concentrations of 5 μ*M*–1000 μ*M* (with very little change in pH) and incubated with the cells for increasing periods of time. The experiments with SNAP were repeated with depleted SNAP (which was added to culture medium and allowed to stand for more than 3 half-life times before being applied to myoblasts) in order to deplete the parent compound of NO, yielding a negative control that can be tested for nonspecific actions of *S*-nitrosothiols on cells. Cell viability was assessed after the addition of the RNS donors and all of the other reagents by Trypan Blue (TBP) staining, and viability was at least 90% at the beginning and at the end of each experiment. (ii) Proteasome inhibitor Z-Leu-Leu-Leu-aldehyde (MG-132) was obtained from Alexis Biochemicals (San Diego, CA, USA). The cells were pretreated with 100 μ*M* of MG-132 (52) for 1.5 h prior to the beginning of the experiment. (iii) Tyrosine nitration inhibitor and antioxidant Epigallocatechine-3-gallate (EGCG) (44) was obtained from Sigma-Aldrich. The cells were pretreated with 100 μ*M* EGCG (35) for 6 h prior to the beginning of the experiment.

Preparation of total cell lysates and subcellular fractions

For total lysate preparation, the cells were lysed in lysis buffer (20 m*M* Tris/HCl, 150 m*M* NaCl, 2 m*M* EDTA, IGEPAL 0.5% v/v, 0.5 m*M* PMSF, 1 μg/ml leupeptin). The lysates were cleared by centrifugation at 10,000 *g* for 3 min. For nuclear protein extraction, cells were lysed as above. Following centrifugation, the pellets were washed in lysis buffer and resuspended in nuclear extraction buffer (HEPES 20 m*M*, $MgCl_2$ 1.5 m*M*, NaCl 420 m*M*, EDTA 0.2 m*M*, DTT 1 m*M*, PMSF 0.5 m*M*, leupeptin 1 μg/ml). After 30 min on ice, followed by brief centrifugation, the supernatants, containing nuclear proteins were collected. Protein concentrations in the samples were determined by Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

SDS-PAGE and Western blot analysis were performed as described (3, 35); 10 μg samples were run in each lane. The equality of sample loading in each lane was confirmed by Coomassie Brilliant Blue gel staining and by Ponceau Red membrane staining following the blotting. A portion of blots was stripped and reblotted with anti-actin antibody to assess the levels of this housekeeping protein in comparison to other proteins tested. Primary antibodies used were: rabbit anti-p65 NF-κB subunit antibody, rabbit anti-I-κB α antibody, mouse anti-phosphorylated on serine 32 I-κB α antibody, goat anti-nitrotyrosine antibody, rabbit anti-inducible nitric oxide synthetase (iNOS), mouse anti-actin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Secondary antibodies were goat anti-rabbit IgG, rabbit anti-goat IgG, and goat anti-mouse IgG (Santa Cruz Biotechnology).

Transient transfection and reporter gene assays

The luciferase reporter construct (pNF-κB-Luc) was purchased from BD Biosciences Clontech (Palo Alto, CA, USA). The β-galactosidase reporter construct was purchased from Promega Corporation (Madison, WI, USA). The cells were grown in 24-well culture plates to ~50% confluence (2 x 10^5 cells per well). On the second day of culture, the cells were co-transfected with the above reporters, using Effectene transfection reagent (Qiagen, Valencia, CA, USA). Subsequently, the cells were maintained at 37°C for 12 h. After that, the growth medium was substituted with serum-free medium in order to minimalize the activation of signal transduction cascades by the growth factors present in the serum. The cells were cultured in the serum-free medium for an additional 24 h, after which the incubations with various substances described above were carried out. Each transfection was repeated three to five times, in triplicates.

Following each experiment, the cells were harvested and lysed using the lysis buffer provided in the Luciferase Reporter Gene Assay kit (BD Biosciences Clontech). The lysates were used for the determination of luciferase activity using the Luciferase Reporter Gene Assay kit, and also for the determination of β-galactosidase activity using the β-

galactosidase Reporter Gene Assay kit (Promega Corporation). The luciferase results were quantified using Anthos Lucy 1 luminometer (Anthos Labtec, Salzburg, Austria). β-Galactosidase results were quantified spectrophotometrically using ELISA Reader Zenyth 2000 (Anthos Labtec). Both luciferase and β-galactosidase activities were normalized to the total amount of protein in the samples (as measured by Bradford assay). Subsequently, luciferase activity was plotted against β-galactosidase activity.

Overexpression of wild-type (WT) and mutant (super-repressor) I-κB α

Plasmids containing genes encoding WT and mutant I-κB α (in which Ser32 and Ser36 are substituted for alanines that cannot undergo phosphorylation, therefore preventing I-κB α degradation and NF-κB canonic activation) were a generous gift of Dr. D. W. Ballard, Vanderbuilt University, Tennessee. The cells were cultured and co-transfected with the plasmids and reporter constructs as described above. The incubations with various substances described above were carried out 36 h after transfection. Each transfection was repeated three to five times, in triplicate. Finally, the cells were lysed as described above. The lysates were used for the determination of luciferase activity and I-κB α protein levels.

Immunoprecipitation and co-immunoprecipitation

Total cell lysates (approximately 200 μg protein) were incubated with 2.5 μg of anti-IKK γ antibody for the IKK activity assay. Cytoplasmic fractions were incubated with 2.5 μg of either anti-I-κB α antibody for determination of I-κB α modification, or anti-p65 antibody for the determination of I-κB α dissociation from NF-κB, at 4°C for 1.5 h. Subsequently, protein A agarose slurry (Santa Cruz Biotechnology Inc.) was added and the mixtures were incubated at 4°C for 1.5 h with agitation. Finally, cytoplasmic fractions were centrifuged at 10,000 *g* for 3 min, and the precipitates were separated and washed. The precipitated IKK was used for IKK activity assay. The precipitated I-κB α was used to determine its nitration or phosphorylation. Both the precipitated p65 and the supernatant left after its precipitation were used to determine I-κB α dissociation from NF-κB.

Confirmation of I-κB α dissociation from NF-κB and determination of I-κB α modification

After immunoprecipitation of p65, the supernatants were subjected to SDS-PAGE and immunoblotted with anti-I-κB α antibody. Strong positive results in the supernatant indicated that I-κB α was dissociated from NF-κB at that particular point in time. After immunoprecipitation of I-κB α, the precipitate was subjected to SDS-PAGE and immunoblotted with either anti-nitrotyrosine or anti-serine-phosphorylated I-κB α antibody to determine the modification of I-κB α.

Nonisotopic in vitro kinase assay

In the cellular system. Cells were treated with 10 μ*M* of either SNAP, PAPA-NONOate, SIN-1, or authentic peroxynitrite for 10 and 20 min. Subsequently, the cells were lysed, and IKK was precipitated. Then, RNS donors were washed out and IKK was resuspended in kinase buffer (20 m*M* Tris/HCl, 20 m*M* β-glycerophosphate, 100 μ*M* sodium orthovanadate, 10 m*M* $MgCl_2$, 50 m*M* NaCl, 1 m*M* DTT, 50 μ*M* ATP, 1 m*M* NaF). Two micrograms of purified recombinant I-κB α protein (Santa Cruz Biotechnology Inc.) were added, and the reaction mixture was incubated at 0°C for 1 h. The reaction was terminated by the addition of Laemmli buffer and sample boiling. The samples were subjected to SDS-PAGE and immunoblotted with anti-serine phosphorylated I-κB α antibody. The level of I-κB α phosphorylation on serine 32 was indicative of the level of IKK activity.

In the cell-free system. The assay was performed in order to determine whether RNS could directly influence the activity of IKK. IKK γ was precipitated from untreated cells as described above. Subsequently, IKK γ was resuspended in PBS and exposed to increasing concentrations (0, 5, 10, 100, and 1000 μ*M*) of RNS donors for 15 min. IKK γ then underwent thorough washing with PBS, followed by second immunoprecipitation in order to remove RNS donors. Subsequently, the precipitated IKK γ was subjected to the IKK activity assay as described above.

Densitometric analysis

Densitometric quantitative analysis of the protein bands detected by Western blot was carried out using Bio1D software (Vilber Lourmat, Torcy, France).

Statistical analysis

Comparisons of the averages of densitometric analyses were made using the unpaired Student's *t* test with significant values set at $p < 0.05$. The results of reporter gene assays were analyzed using Student's *t* test and ANOVA analysis. The results were expressed as average fold increases versus the control values ± SEM.

RESULTS

Nitric oxide induces the classic NF-κB activation pathway in skeletal myoblasts

The cells were exposed to increasing concentrations of NO donors PAPA-NONOate and SNAP (0 μ*M*–100 μ*M*) for 15 min. Nuclear translocation of p65 NF-κB subunit was measured by Western blot in the nuclear fractions from the treated cells. The activation of luciferase reporter gene by NO donors was also examined. At concentrations up to 10 μ*M* PAPA-NONOate and 10 μ*M* SNAP, there was a steady increase in both p65 nuclear translocation (measured for SNAP) and luciferase expression, implying an increase in NF-κB activity. However, at concentrations higher than 10 μ*M*, there was a decrease in both p65 nuclear translocation and luciferase expression (Figs. 1A and 1B). β-Galactosidase activity did not change throughout the experiment (Fig. 1B). Subsequently, the myoblasts were exposed to 10 μ*M* PAPA-NONOate and 10 μ*M* SNAP (which was chosen for subsequent studies as the optimal working concentration) for 60 min, and p65 nuclear translocation and luciferase expression

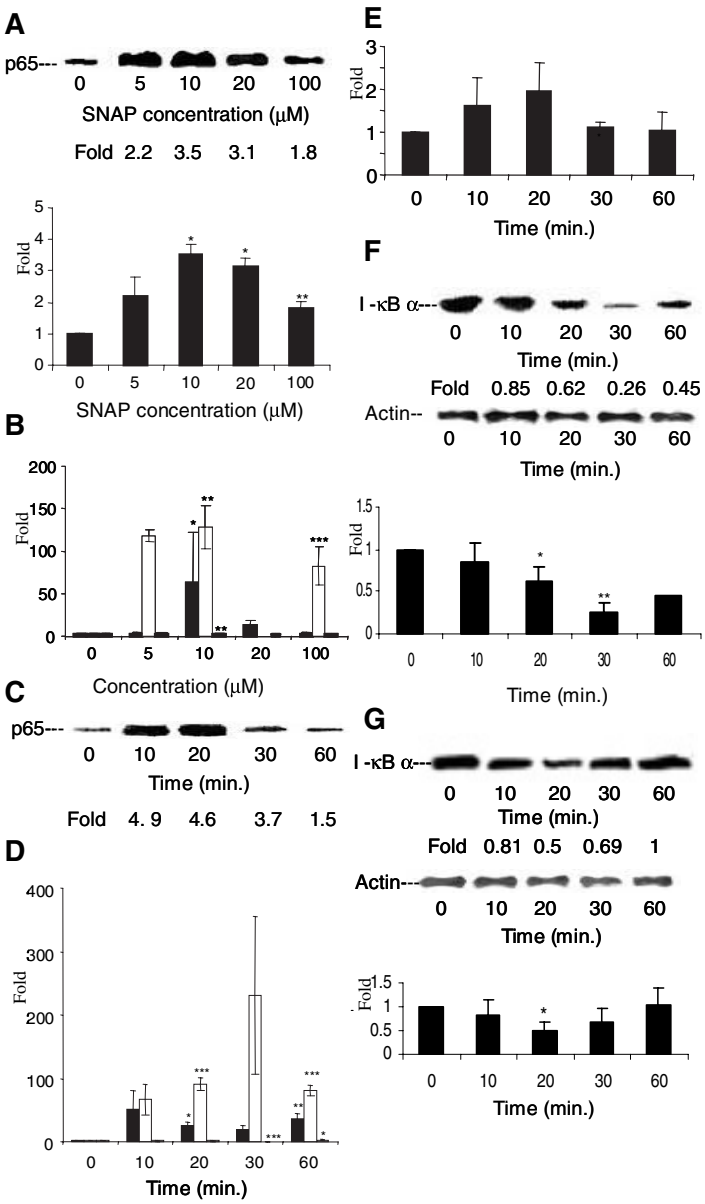

FIG. 1. The effect of NO donors treatment on NF-κB activation in L8 myoblasts. (**A**) L8 cells were treated with increasing concentrations (0, 5, 10, 20, 100 μ*M*) of SNAP for 15 min. p65 nuclear translocation was analyzed by Western blot and quantified densitometrically (see Materials and Methods). **p* < 0.01, ***p* < 0.05 versus control (0 μ*M*). (**B**) The cells were co-transfected with luciferase and β-galactosidase reporter constructs (see Materials and Methods) and treated with increasing concentrations (0, 5, 10, and 100 μ*M*) of SNAP and PAPA-NONOate for 15 min. Luciferase and β-galactosidase activities were analyzed as described in Materials and Methods. **p* < 0.001, ***p* < 0.02, ****p* < 0.05 versus control (0 μ*M*). *Closed bars*, PAPA-NONOate; *open bars*, SNAP; *patterned bars*, β-galactosidase. (**C**) L8 cells were treated with 10 μ*M* SNAP for 10, 20, 30, and 60 min. p65 nuclear translocation was analyzed by Western blot and quantified densitometrically (see Materials and Methods). (**D**) The cells were co- transfected with luciferase and β-galactosidase reporter constructs (see Materials and Methods) and treated with 10 μ*M* of SNAP and PAPA-NONOate for 0, 10, 30, and 60 min. Luciferase and β-galactosidase activities were analyzed as described in Materials and Methods. **p* < 0.01, ***p* < 0.02, ****p* < 0.001 versus control (zero time). *Closed bars*, PAPA-NONOate; *open bars*, SNAP; *patterned bars*, β-galactosidase. (**E**) The cells were transfected with luciferase reporter construct (see Materials and Methods) and treated with 10 μ*M* of depleted SNAP (see Materials and Methods) for 0, 10, 30, and 60 min. Luciferase activity was analyzed as described in Materials and Methods. (**F**) and (**G**) L8 cells were treated with either 10 μ*M* SNAP (**F**) or 10 μ*M* PAPA-NONOate (**G**) for 10, 20, 30, and 60 min. I-κB α degradation and actin levels were analyzed by Western blot and quantified densitometrically (see Materials and Methods). Data are expressed as average fold increase (± SEM for **A**, **B**, **D**) versus control (0 μ*M* for **A** and **B**, zero time for **C**–**G**). The results in (**A**) were derived from four and in (**B**–**G**) from three independent experiments. *In Y axis, fold increase versus untreated control, set as fold 1.

were assessed as a function of time. NO donors caused rapid induction of p65 nuclear translocation (measured for SNAP, Fig. 1C) and luciferase expression after 10 min, with a decline after 20–30 min (Fig. 1D).

β-Galactosidase activity showed little change throughout the experiment (Fig. 1D). In order to determine whether NF-κB activation by SNAP may result from the nonspecific effects of RSNO and not NO, the cells were treated with depleted SNAP, as described in Materials and Methods. This treatment resulted in insignificant activation of luciferase reporter gene (Fig. 1E), as compared to the fold increase in Figure 1D, implying that SNAP functioned as NO donor under these experimental conditions.

To determine whether NO donors in myoblasts caused I-κB α degradation, I-κB α levels were measured in the cytoplasmic fractions by Western blot. 10 μ*M* of both NO donors led to degradation of the cytoplasmic pool of I-κB α after 10–30 min of treatment, with partial I-κB α resynthesis after 60 min (Figs. 1F and 1G). This was consistent with the notion that I-κB α was itself a target gene of NF-κB, and was resynthesized after NF-κB activation (38). This pattern of NF-κB activation was similar to the pattern induced by 4 ng/ml TNF α, a potent inducer of the classic pathway (data not shown). The levels of actin (a housekeeping control protein) did not change throughout this experiment and in the subsequent I-κB α blots (Figs. 2 and 3).

Persistent production of peroxynitrite from SIN-1 induces continuous NF-κB activation in skeletal myoblasts

The cells were exposed to 0 μ*M*–100 μ*M* of peroxynitrite donor SIN-1 between 0 μ*M* and 100 μ*M* for 15 min. Nuclear translocation of NF-κB was measured by Western blot of p65 subunit in the nuclear fractions of the treated cells. Increasing concentrations of SIN-1 caused high and increasing levels of p65 nuclear translocation (Fig. 2A) and a steady in-

crease in luciferase expression (Fig. 2B). β-Galactosidase activity showed little fold change throughout the experiment (Fig. 2B).

Persistent production of peroxynitrite from SIN-1 does not induce the classic NF-κB activation pathway with I-κB α degradation in skeletal myoblasts

Myoblasts were exposed to 10 μ*M* of SIN-1 for 60 min. This caused the rapid induction of p65 nuclear translocation and luciferase expression after 10 min of treatment. There was no significant decrease in NF-κB activation with time (Fig. 2C), in contrast to both NO donors (Figs. 1C and 1D) and TNF-α (data not shown). There was gradual and significant increase in luciferase expression with time (Fig. 2D). β-Galactosidase activity did not change throughout the experiment (Fig. 2D).

The levels of I-κB α were measured in the cytoplasmic fraction of myoblasts by Western blot. Neither 10 μ*M* SIN-1 after 30 min, nor 20 μ*M* SIN-1 after 60 min led to the degradation of I-κB α (Fig. 2E). The levels of actin also did not change (Fig. 2E).

Authentic peroxynitrite induces NF-κB activation in skeletal myoblasts

The cells were exposed to 0 μ*M*–100 μ*M* of authentic peroxynitrite for 15 min. Nuclear translocation of p65 was measured by Western blot, and luciferase expression was assessed. Both p65 nuclear translocation (Fig. 3A) and luciferase expression (Fig. 3B) exhibited significant increase with the increase in peroxynitrite concentrations. β-Galactosidase showed little change throughout the experiment (Fig. 3B).

Authentic peroxynitrite does not induce the classic NF-κB activation pathway with I-κB α degradation in skeletal myoblasts

The myoblasts were exposed to 10 μ*M* of peroxynitrite for 60 min. Peroxynitrite caused rapid induction of both p65 nuclear translocation (Fig. 3C) and luciferase expression (Fig. 3D) after 10 min of treatment. In both cases, there was a gradual decrease in NF-κB activity after 30 min. of treatment. β-Galactosidase activity did not change throughout the experiment (Fig. 3D). Moreover, 10 μ*M* of peroxynitrite did not lead to the degradation of I-κB α (Fig. 3E). In fact, I-κB α levels

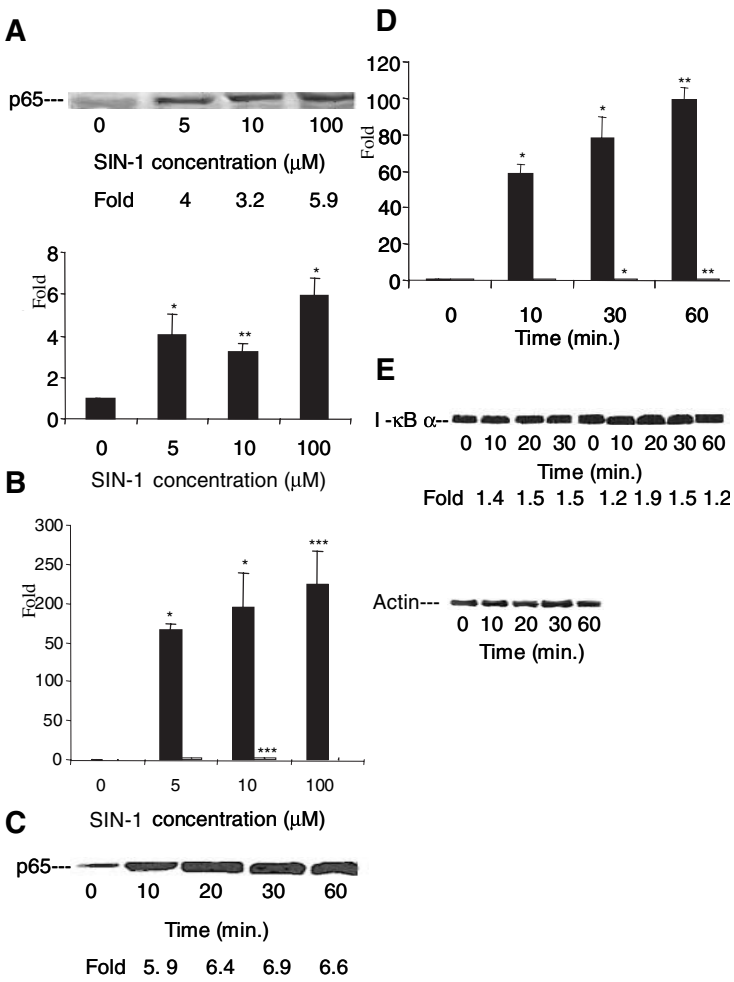

FIG. 2. The effect of SIN-1 treatment on NF-κB activation in L8 myoblasts. (**A**) and (**B**) L8 cells were treated with increasing concentrations (0, 5, 10, and 100 μ*M*) of SIN-1 for 15 min. p65 nuclear translocation (**A**) was analyzed by Western blot and quantified densitometrically (see Materials and Methods). **p* < 0.05, ***p* < 0.001 versus control (0 μ*M*). Luciferase and β-galactosidase activities (**B**) were analyzed as described in Materials and Methods. **p* < 0.01, ***p* < 0.05, ****p* < 0.02 versus control (0 μ*M*). ANOVA between the groups of 5–100 μ*M*: NS (nonsignificant). *Closed bars*, SIN-1; *open bars*, β-galactosidase. (**C**) L8 cells were treated with 10 μ*M* SIN-1 for 10, 20, 30, and 60 min. p65 nuclear translocation was analyzed by Western blot and quantified densitometrically (see Materials and Methods). (**D**) The cells were cotransfected with luciferase and β-galactosidase reporter constructs (see Materials and Methods). Subsequently, L8 cells were treated with 10 μ*M* of SIN-1 for 0, 10, 30, and 60 min. Luciferase and β-galactosidase activities were analyzed as described in Materials and Methods. **p* < 0.01, ***p* < 0.001 versus control (zero time). ANOVA between the groups of 10–60 min: $p = 0.0018$ with a tendency for increase. *Closed bars*, SIN-1; *open bars*, β-galactosidase. (**E**) L8 cells were treated with 10 μ*M* for 10, 20, and 30 min, and with 20 μ*M* SIN-1 for 10, 20, 30, and 60 min. I-κB α degradation and actin levels were analyzed by Western blot and quantified densitometrically (see Materials and Methods). Data are expressed as average fold increase (± SEM for **A**, **B**, **D**) versus control (0 μ*M* for **A** and **B**, zero time for **C**– **G**). The results in **A** were derived from four to five, in **B** and **C** from three, and in **D** and **E** from four independent experiments. *In Y axis, fold increase versus untreated control, set as fold 1.

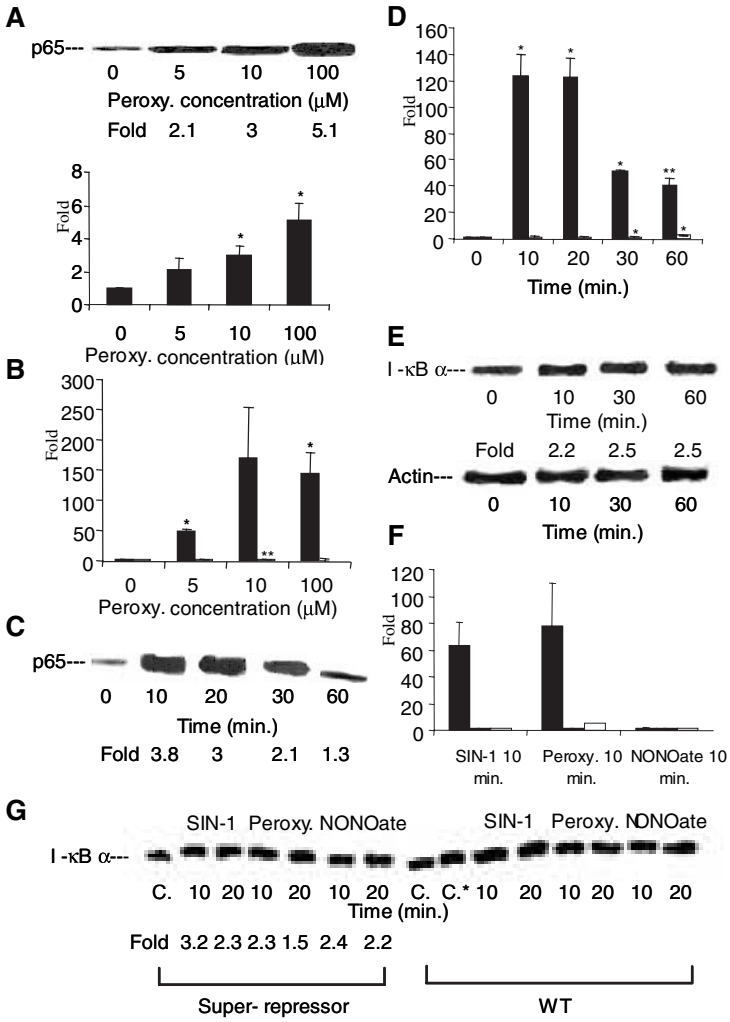

FIG. 3. The effect of the authentic peroxynitrite treatment on NF-κB activation in L8 myoblasts. (**A**) and (**B**) L8 cells were treated with increasing concentrations (0, 5, 10, and 100 μ*M*) of peroxynitrite for 15 min. p65 nuclear translocation (**A**) was analyzed by Western blot and quantified densitometrically (see Materials and Methods). *$p < 0.05$ versus control (0 μ*M*). Luciferase and β-galactosidase activities (**B**) were analyzed as described in Materials and Methods. *$p < 0.01$, **$p < 0.02$ versus control (0 μ*M*). *Closed bars*, peroxynitrite; *open bars*, β-galactosidase. (**C**–**E**) L8 cells were treated with 10 μ*M* peroxynitrite for 0, 10, 20, 30, and 60 min. p65 nuclear translocation (**C**) was analyzed by Western blot and quantified densitometrically (see Materials and Methods). Luciferase and β-galactosidase activities (**D**) were analyzed as described in Materials and Methods. *$p < 0.001$, **$p < 0.01$ versus control (zero time). ANOVA between the groups of 10–60 min: $p = 0.0005$ with a tendency for decrease. *Closed bars*, peroxynitrite; *open bars*, β-galactosidase. I-κB α degradation and actin levels (**E**) were analyzed by Western blot and quantified densitometrically (see Materials and Methods). (**F**) The cells were co-transfected with luciferase and β-galactosidase reporter constructs, and also with plasmids containing the genes for either super-repressor or wild-type forms of I-κB α (see Materials and Methods) and treated with 10 μ*M* of either SNAP, PAPA-NONOate, SIN-1, or authentic peroxynitrite for 0, 10, and 20 min. Luciferase and β-galactosidase activities were analyzed as described in Materials and Methods. *Closed bars*, luciferase activity with super-repressor; *patterned bars*, galactosidase activity with super-repressor; *open bars*, luciferase activity with WT. (**G**) The cells were transfected with plasmids containing the genes for either super-repressor or wild-type forms of I-κB α (see Materials and Methods) and treated with 10 μ*M* of either SNAP, PAPA-NONOate, SIN-1, or authentic peroxynitrite for 0, 10, and 20 min. I-κB α degradation was analyzed by Western blot and quantified densitometrically (see Materials and Methods). *C*, control (not transfected and untreated); *C**, control (transfected and untreated). Data are expressed as average fold increase (± SEM for **A**, **B**, **D**, **F**) versus control (0 μ*M* for **A** and **B**, zero time for **C**–**G**). The results were derived from three independent experiments. *In *y-axis,* fold increase versus untreated control, set as fold 1.

increased with time, probably implying its synthesis *de novo*. The levels of actin did not change (Fig. 3E).

Overexpression of the super-repressor form of I-κB α that could not be phosphorylated and degraded prevented the activation of luciferase reporter construct by PAPA-NONOate (Fig. 3F), implying that NO activated NF-κB through I-κB α degradation. However, it did not prevent the activation of luciferase reporter construct either by SIN- 1 or by authentic peroxynitrite (Fig. 3F), implying that I-κB α degradation after the exposure to peroxynitrite is not a prerequisite for NF-κB activation under these conditions. In contrast to that, overexpression of the wild-type I-κB α prevented NF-κB activation by both NO and peroxynitrite, as determined by luciferase assay (Fig. 3F). As expected, both super-repressor and wild-type I-κB α overexpression resulted in high levels of I-κB α that were not degraded after the treatment with all the agents (Fig. 3G).

Peroxynitrite causes I-κB α dissociation from NF-κB but not its degradation

It was clear that I-κB α was not degraded after exposure to both SIN-1 and the authentic peroxynitrite. In order to determine whether I-κB α dissociated from NF-κB at the time of activation, the cells were treated with 4 ng/ml TNF α (as a positive control for the classic activation), 10 μ*M* of either SNAP, PAPA-NONOate, SIN-1, or peroxynitrite for 0–30 min. Subsequently, the cells were lysed, and NF-κB was precipitated from the lysates using the antibody specific to the amino terminus of p65 subunit. This antibody was selected because NF-κB proteins contain Rel Homology Domains in their N-termini, and these domains interact with I-κB (23, 24). Using this method, bound I-κB α could be co-immunoprecipitated with NF-κB. Consequently, the supernatants

containing I-κB α that remained in the cytoplasm, were immunoblotted with I-κB α -specific antibody.

The cytoplasmic pool of I-κB α became partially degraded after 15 min of treatment with either TNF-α, PAPA-NONOate, or SNAP, and it remained degraded after 30 min of treatment (Fig. 4). These results were consistent with the fact that both TNF-α and NO donors activated NF-κB through the classic pathway in our experimental model.

After either SIN-1 or authentic peroxynitrite, the supernatants contained high levels of I-κB α after either 15 or 30 min of treatment (Fig. 4), suggesting both lack of I-κB α degradation in the cytoplasm and its lack of reassociation with NF-κB. Moreover, after 30 min of treatment with both SIN-1 and authentic peroxynitrite, the cytoplasmic pool of I-κB α was nearly doubled, implying either accumulation or possible I-κB α synthesis *de novo* (Fig. 4).

Proteasome inhibition does not prevent NF-κB activation by peroxynitrite donor SIN-1

Proteasome-dependent degradation of I-κB α is a necessary step in the classic pathway of NF-κB activation (19, 24). Since SIN-1 led to NF-κB activation without I-κB α degradation, it was important to investigate whether proteasome was involved in that atypical activation pathway. The cell culture was pretreated with 100 μ*M* MG-132, a specific and potent proteasome inhibitor, for 1.5 h (lower concentrations proved to be ineffective, while higher concentrations were toxic to the cells as determined by TBP staining, data not shown). Subsequently, the cells were exposed to 10 μ*M* of SIN-1 as a function of time. p65 nuclear translocation and I-κB α levels were assessed by Western blot. It was found that I-κB α was not degraded (Fig. 5B), while NF-κB activation remained high and prolonged (Fig. 5A). In contrast, the treatment of the cells with 10 μ*M* SNAP and MG-132 led to a significant delay in NF-κB nuclear translocation, which became apparent only after 30 min instead of the usual 10 min (data not shown). Accordingly, there was no significant degradation of I-κB α during the measured time frame (data not shown). These observations were consistent with the classic proteasome-dependent activation of NF-κB by SNAP in L8 cells.

Reactive nitrogen species activate IKK

We found that both SIN-1 and authentic peroxynitrite activated NF-κB without I-κB α degradation. Theoretically, the exposure to peroxynitrite could negatively affect I-κB α phosphorylation by IKK. To address this question, IKK activity was measured following SNAP, PAPA- NONOate, SIN-1, or peroxynitrite treatment. The IKK activity assay was run in a cell-free system (as described in Materials and Methods) in order to determine whether RNS could directly activate IKK. The reaction mixture was treated with 0, 10, 100, and 1000 μ*M* SNAP, PAPA-NONOate, and SIN-1 for 15 min, and the activity of the immunoprecipitated IKK was measured as described in Materials and Methods. 10–100 μ*M* NO donors led to an increase in IKK activation, with peak activity at 100 μ*M* SNAP and 10 μ*M* PAPA-NONOate (Figs. 6A and 6B). At 1000 μ*M* SNAP and 100 μ*M* PAPA-NONOate, there was a significant decrease in IKK activation (Figs. 6A and 6B). In contrast to that, SIN-1 led to high levels of IKK activity at 10–1000 μ*M* in the cell-free system (Fig. 6C).

In order to explore how RNS influenced IKK activity in living cells, the cells were treated with 10 μ*M* of either SNAP, PAPA-NONOate, SIN-1, or peroxynitrite for 10 and 20 min, after which the IKK assay was performed as described in Materials and Methods. All RNS agents caused increased levels of IKK activity after 10 min of treatment (Fig. 6D). The less potent IKK activator was PAPA-NONOate. After 20 min of treatment with each agent, there was a decrease in IKK activity, with the exception of PAPA-NONOate (graph in Fig. 6D). The immunoblot is not depicted. This result implied that the activation of IKK by RNS was transient and thus, it could not explain the nontransient activation of NF-κB induced by SIN-1.

The alternative activation of NF-κB by peroxynitrite involves tyrosine nitration

Peroxynitrite is capable of causing the nitration of aromatic amino residues in proteins, mainly tyrosine and tryptophan (5, 12). Tyrosine nitration has been proposed as a signaling event, similarly to phosphorylation (37).

Consequently, we investigated whether tyrosine nitration could be involved in NF-κB activation by peroxynitrite. We used Epigallocatechine-3-gallate (EGCG), a green tea polyphenol capable of directly reacting with tyrosyl radicals and interfering with the formation of 3-nitrotyrosine, which is the main product of tyrosine nitration (37). The cells were pretreated with 100 μ*M* EGCG for 6 h and then exposed to 10 μ*M* SIN-1 as a function of time. p65 nuclear translocation and I-κB α levels were assessed by Western blot. No constitutive NF-κB activation was observed. Instead, NF-κB underwent the classic activation with peak in activity after 20 min, and with I-κB α degradation (Figs. 7A and 7B).

Tyrosine nitration of I-κB α interferes with its serine phosphorylation

I-κB α phosphorylation on serines 32 and 36 is a crucial step in the classic activation of NF-κB. In some cases, tyrosine nitration has been shown to interfere with the phosphorylation of amino residues in the same molecule (35, 37). Therefore, I-κB α phosphorylation as a result of peroxynitrite treatment was measured. The cells were treated with 4 ng/ml TNF-α (as a positive control for the classic activation), or with 10 μ*M* SNAP, 10 μ*M* PAPA-NONOate, 10 μ*M* SIN-1, and 10 μ*M* authentic peroxynitrite for 15 min. Subsequently, I-κB α was precipitated from cell lysates, and its serine phosphorylation was assessed (see Materials and Methods). Next, the blots were stripped and reprobed with anti-nitrotyrosine antibody.

Both TNF-α and NO donors led to I-κB α serine phosphorylation, as could be expected from agents that induced the classic activation pathway (Fig. 8A). In contrast, both SIN-1 and authentic peroxynitrite led I-κB α serine de-phosphorylation (Fig. 8A) and high levels of tyrosine nitration of I-κB α, while both TNF-α and NO donors treatment did not result in the tyrosine nitration of I-κB α (Fig. 8B).

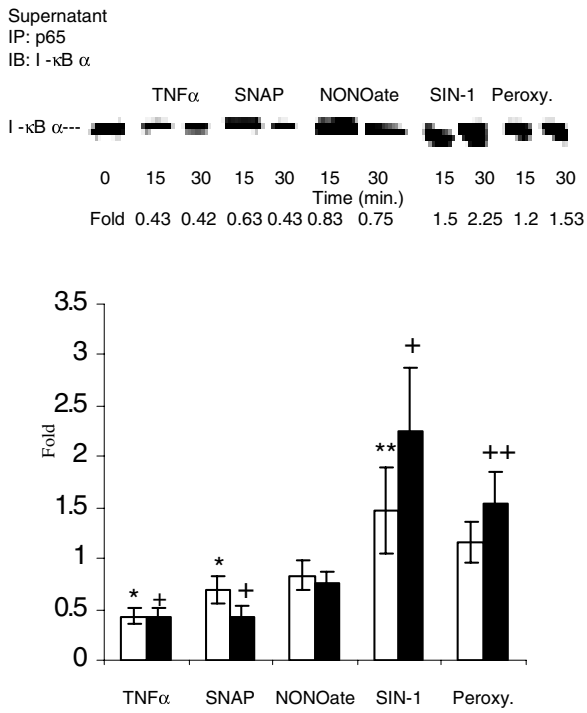

FIG. 4. I-κB α dissociation from NF-κB. L8 cells were treated with either 4 ng/ml TNF-α, or with 10 μ*M* SNAP, 10 μ*M* PAPA-NONOate, 10 μ*M* SIN-1, 10 μ*M* bolus peroxynitrite, for 15 and 30 min. NF-κB was co-immunoprecipitated with I-κB α from total cell lysates at each time point. The supernatants were analyzed for I-κB α by Western blot and quantified densitometrically (see Materials and Methods). Data are expressed as average fold increase ± SEM. **p* < 0.001, ***p* < 0.05, +*p* < 0.001, ++*p* < 0.02 versus control (zero time). *Open bars,* 15 min, *closed bars*, 30 min. The results were derived from three independent experiments. *In *y-axis*, fold increase versus untreated control, set as fold 1.

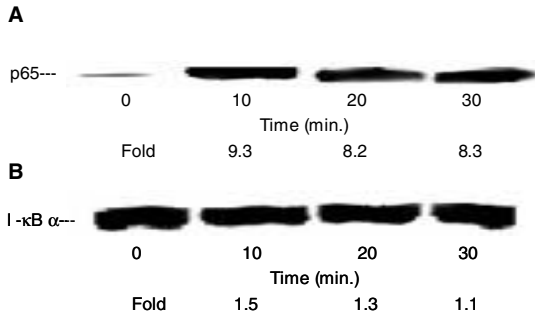

FIG. 5. Proteasome involvement in NF-κB activation by SIN-1. (**A**) and (**B**) L8 cells were pretreated with 100 μ*M* MG-132 for 1.5 h prior to treatment with 10 μ*M* SIN-1 for 10, 20, and 30 min. p65 nuclear translocation (**A**) and I-κB α degradation (**B**) were analyzed by Western blot and quantified densitometrically (see Materials and Methods). Data are expressed as average fold increase versus control (zero time). The results were derived from four independent experiments.

Peroxynitrite donor induces high levels of iNOS expression, which can be markedly reduced by inhibition of tyrosine nitration but not by inhibition of proteasome

The cells were exposed to10 μ*M* SIN-1 for periods of 0, 1, 3, and 24 h. The levels of the pro-inflammatory enzyme, iNOS, which is a target gene of NF-κB (41, 42, 57) were measured in total cell lysates by Western blotting. SIN-1 caused rapid iNOS induction already after 1 h of treatment, and iNOS levels remained high even after 24 h (Fig. 9A). Subsequently the cells were pretreated with either 100 μ*M* EGCG (tyrosine nitration inhibitor) or 100 μ*M* MG-132 (proteasome inhibitor) prior to the exposure to SIN-1. While the pretreatment with MG-132 caused some level of reduction of iNOS expression, pretreatment with EGCG resulted in more pronounced reduction of iNOS expression compared to the untreated control, than MG-132 (Fig. 9B).

DISCUSSION

Recently, RNS have been implicated in a wide variety of pathological conditions (13). In particular, RNS have been shown to modulate inflammatory processes, mainly in leukocytes (1, 35), and in vascular endothelium (8, 9).

Our studies demonstrated that NF-κB could be transiently activated by NO donor PAPA-NONOate and NO/RSNO donor SNAP (which apparently served as primarily NO donor under our experimental conditions, as determined by the experiment with depleted SNAP, Fig. 1E) through the classic pathway that involved IKK activation and I-κB serine phosphorylation and degradation. This activation occurred in a concentration-dependent bell-shape manner (Figs. 1A and 1B). This is consistent with a biphasic NO-dependent mechanism of activation (23). Both NO donors also led to transient time-dependent NF-κB activation (Figs. 1C and 1D), accompanied by gradual I-κB α degradation with subsequent resynthesis (Figs. 1F and 1G). Moreover, overexpression of the mutant super-repressor form of I-κB α resulted in abolishing of NF-κB activation by NO donors, similarly to the overexpression of the wild-type form of I-κB α (Figs. 3F and 3G). Overall, these data suggest that NO in myocytes constitutes an independent and powerful signal for transient NF-κB activation.

The next step in our study was to investigate peroxynitrite-induced NF-κB activation by the use of both peroxynitrite donor SIN-1, and by directly adding peroxynitrite to the cells. Peroxynitrite is a highly reactive compound and a potent oxidizing and nitrating agent (46, 56). It is formed both extracellularly and intracellularly from $NO^{\bullet}$ and $O_2^{-\bullet}$ at diffusion-controlled rates (16) , and is capable of traversing cell membranes and passing several cell diameters (5–20 μm) before its decomposition (46). High levels of peroxynitrite are produced at sites of inflammation (48, 54), in various pathological conditions (4) and in aged tissues (11, 55).

Peroxynitrite induces NF-κB activation in vascular endothelium (8) and murine monocytes (35). According to one study in human monocytes, peroxynitrite (10 and 200 μ*M*) activated NF-κB by nitration of tyrosine residues on I-κB α (35). Another study showed that peroxynitrite donor SIN-1 (0.5 m*M*) activated NF-κB in vascular endothelium cells and caused cyclooxygenase-2 upregulation (8). However, the data on NF-κB activation by peroxynitrite in skeletal muscle are very limited.

SIN-1 is a chemical that decomposes at physiological pH, producing both superoxide and NO, which react immediately

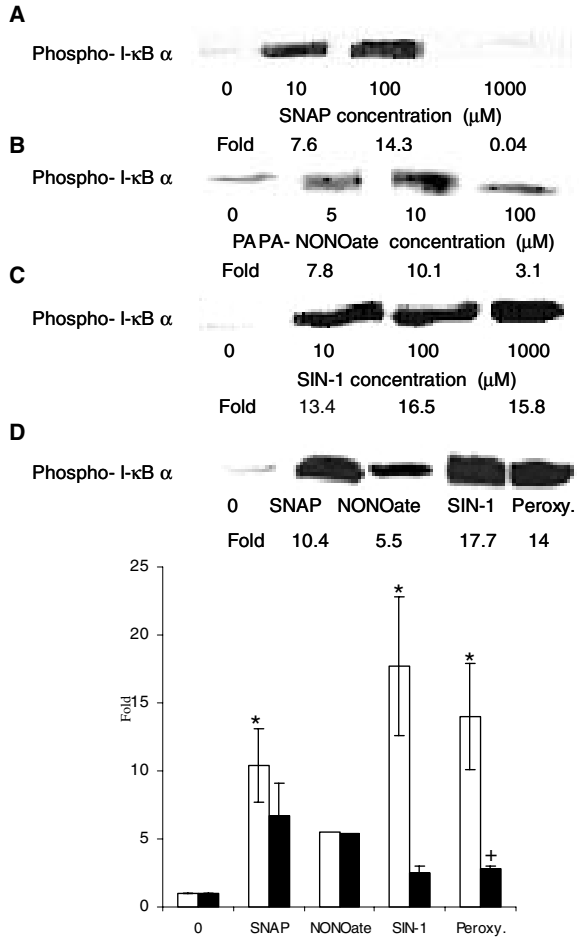

FIG. 6. IKK activation by RNS donors. (**A**) and (**C**) IKK was immunoprecipitated from the lysates of untreated cells, and subsequently treated with 10, 100, and 1000 μ*M* of SNAP (**A**), PAPA-NONOate (**B**), or SIN-1 (**C**) for 15 min in cell-free system. Then, IKK activity assay was performed as described in Materials and Methods. I-κB α serine phosphorylation was assessed by Western blot of the reaction mixture and quantified densitometrically. Data are expressed as average fold increase versus control (no treatment). (**D**) L8 cells were treated with 10 μ*M* SNAP, 10 μ*M* PAPA-NONOate, 10 μ*M* SIN-1, or 10 μ*M* bolus peroxynitrite, for 10 and 20 min in whole cell system. IKK was immunoprecipitated from cell lysates, and IKK activity assay was performed as described in Materials and Methods. I-κB α serine phosphorylation was assessed by Western blot of the reaction mixture and quantified densitometrically. Data are expressed as average fold increase ± SEM. *$p < 0.05$, +$p < 0.001$ versus control (no treatment). *Open bars*, 10 min; *closed bars*, 20 min. The blot is representative of three independent experiments after 10 min of treatment. The results in **A**–**C** were derived from three, and in **D** from three to five independent experiments. *In *y-axis*, fold increase versus untreated control, set as fold 1.

to form peroxynitrite (33, 49). SIN-1 decomposes slowly over a period of 1–2 h, resulting in the prolonged exposure of the cells to relatively stable levels of peroxynitrite (33, 49). In contrast, the half-life of the authentic peroxynitrite under physiological conditions is about 1 sec (46). Therefore, bolus addition of peroxynitrite to the cells results in short-term exposure, whereas the addition of SIN-1 most probably mimics the production of peroxynitrite under physiological and pathological conditions. Consequently, most of the experiments in this study were performed preferentially with SIN-1. NF-κB activation by SIN-1 was concentration-dependent and it increased with the rise in peroxynitrite donor concentrations (Figs. 2A and 2B). Furthermore, with time SIN-1 caused increasing and prolonged p65 nuclear translocation and luciferase expression (Figs. 2C and 2D, respectively). Moreover, peroxynitrite did not cause the degradation of I-κB α (Fig. 2E). Also, overexpression of the super-repressor form of I-κB α did not abolish NF-κB activation by SIN-1, in contrast to the overexpression of the wild-type I-κB α (Figs. 3F and 3G), suggesting that phosphorylation and proteasomal degradation of I-κB was not required for NF-κB activation by SIN-1. Direct addition of authentic peroxynitrite to the cells also resulted in potent activation of NF-κB. Similarly to SIN-1, both p65 nuclear translocation and luciferase expression were directly correlated to the concentrations of peroxynitrite (Figs. 3A and 3B, respectively). NF-κB activity in this case was transient (Figs. 3C and 3D). Similarly to SIN-1, there was no detectable I-κB α degradation (Fig. 3E), and overexpression of the super-repressor form of I-κB α did not abolish NF-κB activation by peroxynitrite (Fig. 3F), suggesting that NF-κB activation by peroxynitrite was not proteasome-dependent.

These results imply that the duration of the exposure to peroxynitrite directly influences the duration of NF-κB activation. SIN-1, which releases peroxynitrite gradually over prolonged periods of time, causes persistent NF-κB activation. Conversely, the short- lived authentic peroxynitrite leads to transient NF-κB activation.

Considering the fact that both SIN-1 and peroxynitrite led to NF-κB activation without I-κB α degradation, it was important to ascertain whether I-κB α was dissociated from NF-κB. Indeed, several alternative activation mechanisms recently described involved I-κB α dissociation from NF-κB without its degradation (32, 43, 53, 60). Both NO donors and TNFα caused I-κB α degradation in the cytoplasm following NF-κB activation. However, both SIN-1 and peroxynitrite caused rapid I-κB α dissociation from NF-κB. The dissociated I-κB α accumulated in the cytoplasm but did not reassociate with NF-κB (Fig. 4).

In order to elucidate the role of the proteasome in NF-κB activation by SIN-1, we partially inhibited the proteasome in the cell culture using the specific proteasome inhibitor MG-132. The proteasome inhibition led to a significant delay and decrease in NF-κB activation and I-κB α degradation after SNAP treatment (unpublished data). However, the proteasome did not participate in NF-κB activation by SIN-1, since its inhibition did not lead to a decrease in NF-κB activation after SIN-1 treatment (Figs. 5A and 5B). This observation raised the possibility that peroxynitrite induced a modification that prevented the recognition of I-κB α by the ubiquitin–proteasome system. This hypothetic modification could also cause I-κB α dissociation from NF-κB. Thus, we hypothesized that either I-κB α or IKK upstream of it, might be modified by peroxynitrite, resulting in the lack of I-κB α recognition by the ubiquitin–proteasome pathway. To test this hypothesis, we investigated whether IKK was directly influenced by RNS donors. IKK activity was measured in an isolated cell-free system after the exposure of IKK to RNS

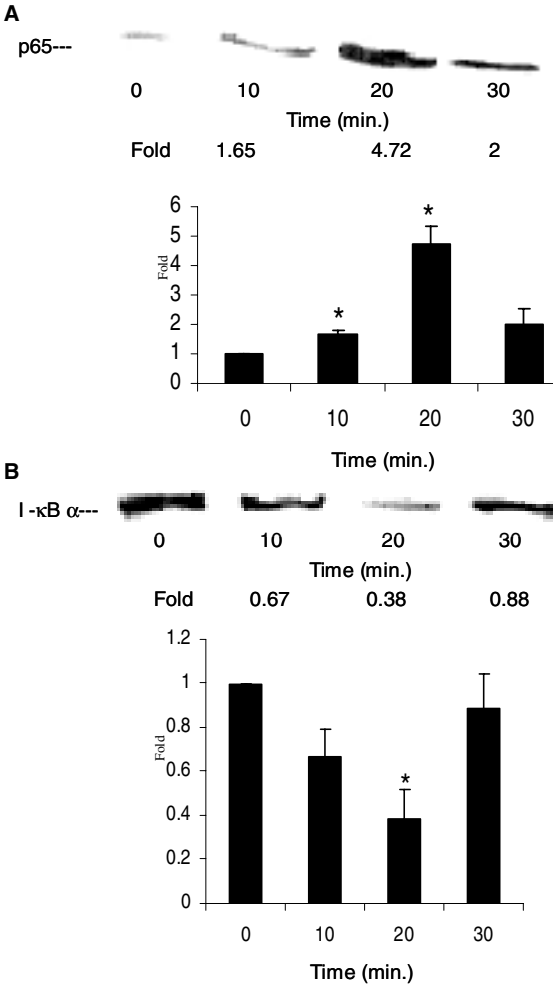

Fig. 7. The involvement of tyrosine nitration in NF-κB activation by RNS donors. (**A**) and (**B**) L8 cells were pretreated with 100 μ*M* EGCG for 6 h prior to treatment with 10 μ*M* SIN-1 for 10, 20, and 30 min. p65 nuclear translocation (**A**) and I-κB α degradation (**B**) were analyzed by Western blot and quantified densitometrically (see Materials and Methods). Data are expressed as average fold increase ± SEM. **p* < 0.01 versus control (zero time). The results were derived from six (**A**) or five (**B**) independent experiments. *In *y-axis,* fold increase versus untreated control, set as fold 1.

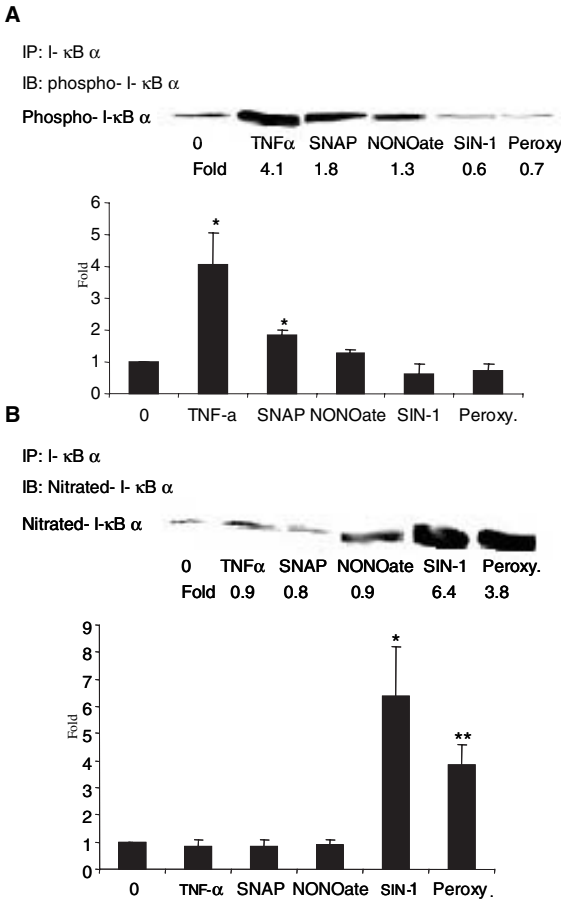

FIG. 8. Tyrosine nitration of I-κB α interferes with its serine phosphorylation. (**A**) and (**B**) L8 cells were treated either with 4 ng/ml TNF-α, or with 10 μ*M* SNAP, 10 μ*M* PAPA-NONOate, 10 μ*M* SIN-1, and 10 μ*M* bolus peroxynitrite for 20 min. I-κB α serine phosphorylation (**A**) **p* < 0.01, ***p* < 0.02 versus control (no treatment) and tyrosine nitration (**B**) **p* < 0.05, ***p*< 0.01 versus control (no treatment)) were assessed as described in Materials and Methods and quantified densitometrically. Data are expressed as average fold increase ± SEM. The results were derived from four to five (**A**) and five (**B**) independent experiments. *In *y-axis,* fold increase versus untreated control, set as fold 1.

donors. PAPA-NONOate, SNAP, and SIN-1 were capable of directly activating the inactive IKK (Figs. 6A–6C). NO donors activated IKK in the concentration range of 10–100 μ*M*. However, at higher concentrations of NO donors (100 μ*M*–1 m*M*), there was a significant decrease in IKK activity (Figs. 6A–6C), which was fully consistent with the previous finding of NF-κB inhibition by high concentrations of NO donors (ranging from 100 μ*M* to 1 m*M*), as reported by the Janssen-Heininger group (47). According to their recent study, RSNO caused a dose-dependent inhibition of IKK, in lung epithelial cells (C10) and in Jurkat T cells, which was associated with *S*-nitrosylation of the IKK complex.

Conversely, SIN-1 led to high levels of IKK activity even at the high concentrations of 100–1000 μ*M* (Fig. 6C). Furthermore, when the living cells were treated with 10 μ*M* of either NO donors, SIN-1, or authentic peroxynitrite, each agent, including SIN-1 and peroxynitrite, led to high and transient IKK activity in the living cells (Fig. 6D). Therefore, the limited duration of IKK activation could not explain the nontransient activation of NF-κB by SIN-1. Moreover, since either SIN-1 or peroxynitrite increased IKK activity, but prevented I-κB α degradation, it was probable that the hypothetical peroxynitrite-induced modification occurred downstream to IKK.

Peroxynitrite has been demonstrated to trigger nitration of proteins, yielding 3-nitrotyrosine (59). Tyrosine nitration has been observed in various pathological conditions. It has been proposed to represent one of the major pathophysiological protein modifications (59) and to modulate signal transduction (37).

I-κB α has been also the main target of modifications in the few alternative pathways of NF-κB activation that have been described (38, 43, 53). These observations led us to the

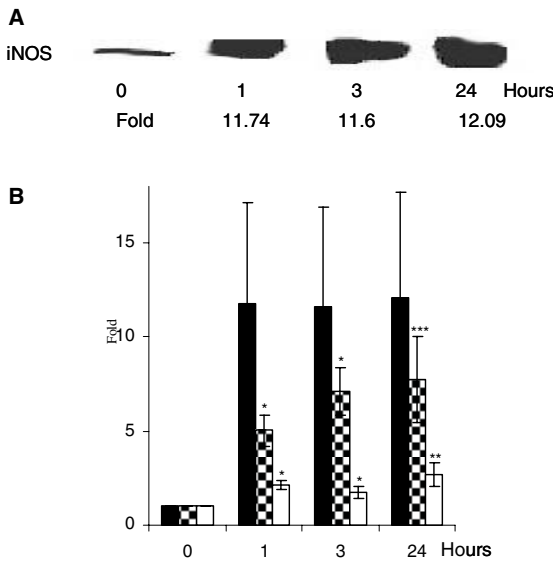

FIG. 9. iNOS induction by SIN-1. (**A**) L8 cells were treated with 10 μ*M* of SIN-1 for 1, 3, and 24 h. iNOS induction was assessed by Western blot and quantified densitometrically (see Materials and Methods). (**B**) L8 cells were pretreated with either 100 μ*M* MG-132 for 1.5 h or 100 μ*M* of EGCG for 6 h and treated with 10 μ*M* of SIN-1 for 1, 3, and 24 h. iNOS induction was assessed by Western blot and quantified densitometrically (the blots are not shown). Data are expressed as average fold increase in iNOS versus control (no treatment) ± SEM (in **B**). *$p < 0.001$, **$p < 0.05$, ***$p < 0.02$ versus control (no treatment). *Closed bars*, SIN-1; *patterned bars*, SIN-1 + MG-132; *open bars*, SIN-1 + EGCG. The results were derived from three to seven independent experiments. *>In *y-axis*, fold increase versus untreated control, set as fold 1.

TABLE 1. THE OUTCOMES OF RNS TREATMENTS ON NF-κB ACTIVATION IN L8 MYOBLASTS

Endpoints Intervention	*p65 nuclear translocation*	*Luciferase activation*	*I-κB α degradation*	*NF-κB inhibition by SuperR`*	*I-κB α dissociation from NF-κB*	*IKK activation*	*I-κB α serine phosphorylation*	*I-κB α tyrosine nitration*	*iNOS expression*
PAPA-NONOate, Increasing concentrations	Biphasic (Bell shape)	Biphasic (Bell shape)				Biphasic (Bell shape)			
PAPA-NONOate, time-dependent	Transient	Transient	Transient	Inhibition	Dissociation and degradation	Transient	Phosphorylation	Insignificant	
SNAP, Increasing concentrations	Biphasic (Bell shape)	Biphasic (Bell shape)				Biphasic (Bell shape)			
SNAP, time-dependent	Transient	Transient	Transient	Inhibition	Dissociation and degradation	Transient	Phosphorylation	Insignificant	
SIN-1 Increasing concentrations	Increasing	Increasing				Constitutive			
SIN-1 time-dependent	Constitutive	Constitutive	No degradation	No inhibition	Dissociation degradation	Transient	Insignificant	Nitration	Very high
Peroxynitrite, Increasing concentrations	Increasing	Increasing							
Peroxynitrite, time-dependent	Transient	Transient	No degrada-= tion	No inhibition	Dissociation no degradation		Insignificant	Nitration	
SNAP + MG-132	No translocation		No degradation						
SIN-1+ MG-132	Constitutive		No degradation						High
SNAP + EGCG	No translocation		No degradation						
SIN-1 + EGCG	Transient		Transient						Low

possibility that peroxynitrite could induce a modification of I-κB α (such as tyrosine nitration) which, in turn, could cause the alternative activation of NF-κB by peroxynitrite. To test this hypothesis, nitrotyrosine formation was inhibited by the use of the nitration inhibitor, EGCG, prior to the treatment of the cells with SIN-1. EGCG is an antioxidant that reacts with tyrosyl radicals, thus preventing their reaction with RNS and subsequent formation of nitrotyrosine (35). EGCG in combination with SIN-1 led to the induction of the classic activation with transient NF-κB activation and I-κB α degradation (Figs.7A and 7B). This shows that tyrosine nitration indeed is involved in and is vital for the alternative activation of NF-κB by peroxynitrite. In addition, both SIN-1 and authentic peroxynitrite, in contrast to both TNF-α and NO donors, caused I-κB α serine dephosphorylation (Fig. 8A) and high I-κB α tyrosine nitration (Fig. 8B). Therefore, peroxynitrite caused tyrosine nitration of I-κBα, which probably interfered with its serine phosphorylation. Since serine phosphorylation marks I-κB α for proteasomal degradation, once this phosphorylation is abolished, I-κB α cannot be recognized by the specific ubiquitin ligases and therefore cannot undergo proteasomal degradation. Moreover, in some cases tyrosine nitration may induce conformational change in the protein (37), which may explain I-κB α dissociation from NF-κB.

Furthermore, our study implies that the nitrated I-κB α cannot be degraded in the proteasome, and instead accumulates in the cytoplasm. Tyrosine-nitrated I-κB α dissociates from NF-κB probably as a result of a conformational change or steric hindrance imposed by the nitration and dephosphorylation. In this fashion, tyrosine nitration of I-κB α leads to the constitutive activation of NF-κB and the loss of the negative feedback on such activation, resulting in continuous production of inflammatory mediators.

Theoretically, as NF-κB activity continues, new I-κB α would be synthesized and, if the presence of peroxynitrite persists, as in the case of SIN-1, this new I-κB α would also become a target for tyrosine nitration. In this manner, NF-κB cannot be removed from the nucleus, and its activity will not significantly decrease with time. One possible implication of this phenomenon is that in tissues where high concentrations of peroxynitrite are produced for protracted periods, NF-κB may exit the autoinhibitory loop and enter the vicious cycle of constitutive activation. Therefore, high levels of inflammatory mediators such as iNOS (Fig. 9) may be produced, resulting in high levels of NO which, after reacting with intracellular superoxide, may lead to even higher peroxynitrite levels, thus contributing to the vicious cycle of inflammation proposed above. Short-term exposure to peroxynitrite, such as bolus addition of authentic peroxynitrite to the cell culture, also results in I-κB α nitration and dephosphorylation. However, since this type of exposure is limited, the newly synthesized I-κB α would not become nitrated, and NF-κB activation would be transient. The findings of this study are summarized in Table 1.

ACKNOWLEDGMENTS

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ABBREVIATIONS

β-Gal, β-Galactosidase; EGCG, epigallocatechine-3-gallate; I-κB, inhibitor of κB; IB, immunoblotting; IP, immunoprecipitation; IKK, inhibitor of κB kinase; Luc, luciferase; MG-132, Z-leucine-leucine-leucine aldehyde; NF-κB, nuclear factor-κB; NS, nonsignificant; PAPA-NONOate, 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine; RNS, reactive nitrogen species; RSNO, *S*-nitrosothiols; SIN-1, 3-morpholino-sydnonimine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TNF-α, tumor necrosis factor α; WT, wild type.

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