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Published in final edited form as:

**Title:** Peroxynitrite is a potent inhibitor of NF- $\kappa$ B activation triggered by inflammatory stimuli in cardiac and endothelial cell lines.

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**Journal:** The Journal of biological chemistry

**Year:** 2005 Oct 14

**Volume:** 280

**Issue:** 41

**Pages:** 34878-87

**DOI:** 10.1074/jbc.M501977200

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Published in final edited form as:

*J Biol Chem.* 2005 October 14; 280(41): 34878–34887.

## PEROXYNITRITE IS A POTENT INHIBITOR OF NF- $\kappa$ B ACTIVATION TRIGGERED BY INFLAMMATORY STIMULI IN CARDIAC AND ENDOTHELIAL CELL LINES

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### Abstract

Peroxynitrite is a potent oxidant and nitrating species proposed as a direct effector of myocardial damage in numerous cardiac pathologies. Whether peroxynitrite also acts indirectly, by modulating cell signal transduction in the myocardium, has not been investigated. We therefore examined a possible role of peroxynitrite on the activation of NF- $\kappa$  B, a crucial pro-inflammatory transcription factor, in cultured H9C2 cardiomyocytes. H9C2 cells were stimulated with TNF  $\alpha$  or LPS following a brief (20 min) exposure to peroxynitrite. NF- $\kappa$  B activation (phosphorylation and degradation of its inhibitor I  $\kappa$  B  $\alpha$ , nuclear translocation of NF- $\kappa$  B p65 and NF- $\kappa$  B DNA binding) triggered by LPS or TNF  $\alpha$  was abrogated by peroxynitrite. Peroxynitrite also inhibited NF- $\kappa$  B in 2 human endothelial cell lines activated with TNF  $\alpha$  or IL-1  $\beta$ . These effects were related to oxidative, but not nitrative chemistry, being still observed while nitration was suppressed by epicatechin. The mechanism of NF- $\kappa$  B inhibition by peroxynitrite was a complete blockade of phosphorylation and activation of the upstream kinase IKK  $\beta$ , required for canonical, pro-inflammatory NF- $\kappa$  B activation. At the same time, peroxynitrite activated phosphorylation of NIK and IKK  $\alpha$ , considered as part of an alternative, non canonical NF- $\kappa$  B activation pathway. Suppression of IKK  $\beta$ -dependent NF- $\kappa$  B activation translated into a marked inhibition of the transcription of NF- $\kappa$  B dependent genes by peroxynitrite. Thus, peroxynitrite has a dual effect on NF- $\kappa$  B, inhibiting canonical IKK  $\beta$ -dependent NF- $\kappa$  B activation while activating NIK and IKK  $\alpha$  phosphorylation, which suggests its involvement in an alternative pathway of NF- $\kappa$  B activation. These findings offer new perspectives in the understanding of the relationships between redox stress and inflammation.

NF- $\kappa$  B is a crucial transcription factor activating inflammatory and anti-apoptotic genes in response to immunostimulation. NF- $\kappa$  B is a family of dimeric proteins which include NF- $\kappa$  B1 (p50 and its precursor p105), NF- $\kappa$  B2 (p52 and its precursor p100), p65 (RelA), RelB and c-Rel, the commonest dimer being formed from a p50 subunit and a p65 subunit. NF- $\kappa$  B is normally held in the cytoplasm in an inactive form, bound to inhibitory proteins, the I $\kappa$ Bs. The critical step in NF- $\kappa$  B activation relies in its dissociation from the I $\kappa$ B protein, which results from stimulus-induced phosphorylation of I $\kappa$ B, followed by its polyubiquitination and degradation by the proteasome. I $\kappa$ Bs are phosphorylated by a protein kinase complex, I $\kappa$ B kinase (IKK), composed of a heterodimer of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , associated

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with a dimer of a regulatory subunit, IKK $\gamma$  or NEMO, NF- $\kappa$ B Essential Modulator (for review, see (1,2)). Two distinct pathways of NF- $\kappa$ B activation have been described. In the first one, termed the *canonical pathway*, IKK $\beta$  is activated by an unknown upstream activator via regulatory IKK $\gamma$ , and then phosphorylates I $\kappa$ B. This pathway is responsible for activation of inflammatory responses and the transcription of genes which protect against apoptosis (3–6). In addition to inflammatory signals, a number of reports have indicated that canonical NF- $\kappa$ B activation is also redox sensitive, as it can be activated in a number of different cell types by oxidants and free radicals (7,8). The second pathway of NF- $\kappa$ B activation, termed the *non canonical pathway*, is independent from IKK $\gamma$  and IKK $\beta$ , but is based on NF- $\kappa$ B inducing kinase (NIK) and IKK $\alpha$ -dependent processing of NF- $\kappa$ B p100 to p52, a pathway crucial for B cell maturation and lymphoid organ development (9–12). In contrast to the canonical pathway, it is currently unknown whether this alternate pathway is responsive to redox stress.

Peroxynitrite (PN) is a highly reactive oxidant and nitrating species formed by the reaction of nitric oxide (NO) with superoxide (O $_2^-$ ) (13). PN generation is favored when the production of both NO and O $_2^-$  is enhanced, as may occur during inflammation, circulatory shock and reperfusion injury (14). In the heart, PN generation occurs during myocardial infarction (15), heart failure (16) and cardiomyopathy due to anthracyclines (17). In such conditions, it is unknown whether PN only acts as a direct cytotoxic effector, or may have further, indirect effects related to the modulation of redox sensitive cell signal transduction pathways such as NF- $\kappa$ B. It is also noteworthy that cardiomyocytes are not only exposed to PN in these circumstances, but also to additional inflammatory stimuli such as pro-inflammatory cytokines. Whether PN may alter the cell response to these stimuli has not been evaluated. The present study was therefore designed to explore a possible crosstalk between PN and inflammatory stimuli (TNF $\alpha$  and LPS) at the level of NF- $\kappa$ B signaling in cultured cardiomyocytes and endothelial cells. Our findings demonstrate that PN blocks IKK $\beta$  phosphorylation and activation, thereby preventing nuclear translocation of NF- $\kappa$ B and inflammatory gene transcription in response to immune stimulation. In parallel, PN strongly activates the phosphorylation of NIK and IKK $\alpha$ , whether or not the cells are exposed to immune stimuli. Thus, PN has a dual effect on NF- $\kappa$ B, being an inhibitor of canonical IKK $\beta$ -dependent NF- $\kappa$ B activation pathway while simultaneously activating the phosphorylation of NIK and IKK $\alpha$ , suggesting its involvement in an alternative pathway of NF- $\kappa$ B activation.

## MATERIAL AND METHODS

### Cell culture conditions and reagents

H9C2 cells, a clonal line derived from rat heart (ATCC, Manassas, VA) and human macrovascular endothelial cells (EAhy 926, a generous donation of Prof. D. Hayoz, Division of Hypertension, Lausanne University Hospital) were grown (5% CO $_2$ , 37 °C) in DMEM (Gibco BRL, Invitrogen, Basel, Switzerland) supplemented with 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The human microvascular endothelial cell line HMEC-1, a generous gift from Dr F.J. Candal, Center of Disease Control and Prevention, Atlanta, GA, were cultured (5% CO $_2$ , 37 °C) in MCDB 131 medium (GibcoBRL) enriched with 10% FBS, 50 units/ml penicillin, 50  $\mu$ g/ml streptomycin, and 0.01 mg/ml epidermal growth factor (EGF) (Roche; Basel, Switzerland). Cells were cultured to 80 percent confluence and were serum-starved 24h before the experiments. Unless otherwise specified, all the reagents used in this study were obtained from Sigma-Aldrich (Fluka, Buchs, Switzerland).

### Stimulation with PN, LPS, TNF $\alpha$ and IL-1 $\beta$

PN (Calbiochem), was synthesized as described previously (18). Stocks of PN were stored in 0.4 M NaOH at -80°C. Prior to experimentation, the concentration of PN was determined from its absorption at 302 nm (extinction coefficient = 1670 M $^{-1}$  cm $^{-1}$ ) (13). For stimulation with

PN, cells were washed once and then covered with PBS-glucose buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 90 mM NaCl, 5 mM KCl, 0.8 mM MgCl<sub>2</sub>, 0.2 mM CaCl<sub>2</sub>, 5 mM glucose, pH 7.4). PN was delivered as a single bolus at a 1:100 dilution. The final concentration of PN was 10–250 μM depending on the experimental conditions, in agreement with the concentrations of PN used in previous studies from our laboratory (19), and from other investigators (20–23). Such concentrations of PN are physiologically relevant, as it has been estimated that the rate of PN generation may reach up to 1mM/min in an inflamed organ (lung) in vivo (24). Cells were exposed to PN for 20 min, after which they were washed and replaced in culture medium for stimulation with either 1 μg/ml LPS (E. Coli O127:B8, Sigma Chemicals), 20 ng/ml TNFα (Socochim SA, Lausanne, Switzerland) or 1 ng/ml IL-1β (PreproTech, London, UK) for the indicated times. Control experiments were performed using decomposed PN (DP) in NaOH, obtained by leaving the PN solution overnight at room temperature, after which PN is completely decomposed as determined spectrophotometrically (19,25). In some experiments, hydrogen peroxide instead of PN was used to pretreat the cells before immunostimulation.

### Preparation of cytoplasmic and nuclear extracts

Cells were scraped into hypotonic buffer (HEPES 10 mM, pH 7.9, EDTA 0.1 mM, EGTA 0.1 mM, KCl 10 mM, DTT 1 mM, PMSF 1 mM, Na<sub>3</sub>VO<sub>4</sub> 1 mM). After addition of NP-40 (0.625 %), the tubes were centrifuged and supernatants containing the cytoplasmic proteins were stored at –70°C. Pellets were resuspended in hypertonic buffer (HEPES 20 mM, pH 7.9; NaCl 0.4 M, EDTA 1 mM, EGTA 1 mM, DTT 1 mM, PMSF 1 mM). After centrifugation, supernatants (nuclear extract) were collected and stored at –70°C. Amount of proteins was quantified by BCA assay.

### SDS Page and Western immunoblotting

30 μg of cytoplasmic or 10 μg of nuclear proteins were separated by SDS-Page, transferred to nitrocellulose membranes and blocked for 1 h at room temperature in PBS/0.1% Tween/3% nonfat dry milk. For nuclear extracts, the membrane was incubated with a rabbit anti-NF-κB RelA/p65 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and for cytoplasmic extracts, the membrane was incubated with appropriate dilutions of primary anti-IκBα, anti-phospho-IκBα (both from Santa Cruz), anti-IKKα, anti-IKKβ, anti-phospho-IKKα/β, anti-phospho-IKKα, anti-NIK, anti-phospho NIK, anti-NF-κ B RelA/p65, anti-α-tubulin (all from Cell Signaling, Beverly, MA) or anti-nitrotyrosine antibody (Upstate biotechnology, Lake Placid, NY), followed by incubation with a 1:5000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). The immunoblot signal was visualized using enhanced chemiluminescence (ECL, Amersham Biosciences).

### Electromobility shift assay (EMSA)

An NF-κ B oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGG-3') was labeled with α-<sup>32</sup>PdCTP using the Klenow enzyme (Roche). 10 μg of nuclear proteins were incubated with EMSA buffer (20 mM Hepes pH 7.9, 50 mM KCl, 0.5 mM EDTA, NP40 0.1 %, BSA 1 mg/ml, Glycerol 5%), 170 μg/ml poly dIdC and the probe for 20 min at room temperature. Samples were resolved on a non-denaturing polyacrylamide gel. Gels were transferred to Whatman 3M paper, dried under vacuum, and exposed to photographic film at –70°C with intensifying screens. Densitometric analysis of autoradiographs were performed using a Personal Densitometer and TotalLab Software.

### NF-κB gene reporter gene assay

Cells were transiently transfected with 1 μg of a multimeric NF-κB pGL2 luciferase vector and 0.1 μg of the *Renilla* pRL-TK vector (kind gifts from Prof. T. Calandra, Department of

Infectious Diseases, Lausanne University Hospital) using Lipofectamine 2000 (Invitrogen, Basel, Switzerland). The media was changed 6 h after transfection and the cells were grown overnight in fresh complete media. Luciferase activity was determined using a standard kit (Dual-Luciferase Reporter assay system, Promega Biosciences Inc, San Luis Obispo, CA). The activity of the NF- $\kappa$ B reporter luciferase was standardized to that of Renilla luciferase.

### Immunoprecipitation and IKK kinase assay

Cells were lysed in LB (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 0.1% NP-40, 10  $\mu$ g/ml Leupeptin, 10  $\mu$ g/ml Aprotinin, 1  $\mu$ g/ml Pepstatin, 0.5 mM DTT, 100  $\mu$ M NaF). IKK was immunoprecipitated by incubation of 200  $\mu$ g of protein with an anti-IKK $\beta$  antibody overnight at 4°C, followed by addition of Protein A Sepharose beads CL-4B (Amersham) for 3 h. Precipitates were washed 5x with LB and 3x with Kinase Buffer (20 mM HEPES pH 7.9, 20 mM  $\beta$ -glycerophosphate, 1 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 2 mM NaF, 1 mM DTT). The kinase reaction was performed by adding 1  $\mu$ g of recombinant GST-I $\kappa$ B $\alpha$  (aminoacids 1–54, MTR Scientific, Ijamsville, MD) and 5  $\mu$ Ci  $\gamma$ -<sup>32</sup>PATP and incubating 30' à 30°C. Reactions were stopped by addition of 2xLaemmli sample buffer. Proteins were separated on a 12% polyacrylamide gel, and gels were fixed, dried and examined by autoradiography.

### RNA isolation and polymerase chain reaction

Cells were stimulated with LPS or TNF $\alpha$  for 2 h and total RNA was isolated by TRIZOL (Invitrogen). RNA was reverse-transcribed to cDNA and amplified by PCR using the One Step RT-PCR kit from Qiagen (Qiagen AG, Hombrechtikon, Switzerland) with a Tpersonal Thermocycler (Biometra, Goettingen, Germany). The sequences of the primer pairs were as follows: rat iNOS (sense, 5'-CTGCATGGAACAGTATAAGGCAAAC-3'; antisense, 5'-AGACAGTTTCTGGTTCGATGTCATGA-3'); rat TNF  $\alpha$  (sense, 5'-TCTGTCTACTGAACTTCGGGGTGAT-3'; antisense, 5'-CAGCCTTGCCCTTGAAGAGAACC-3'); rat GAPDH, used as an internal control (sense, 5'-ACCACAGTCCATGCCATCAC-3'; antisense : 5'-TCCACCACCTGTTGCTGTA-3'). PCR products were run by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

### Measurement of cell viability by MTT assay

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan. Cells were stimulated for the indicated periods, and then incubated for 60 minutes with MTT (0.5 mg/ml), followed by aspiration and solubilisation of the cells in 100 $\mu$ l DMSO, and absorbance was measured at 540 nm.

### Immunocytochemistry

Cells plated on glass coverslips were treated with PN (20 min), and then fixed with 10% paraformaldehyde. Cells were then incubated with polyclonal rabbit anti-nitrotyrosine (Upstate) at room temperature for 2h. A standard immunostaining protocol was then followed using Vectastain ABC kit from Vecor Laboratories (Burlingame, CA).

### Statistical analysis

Densitometric analyses of blots are shown as mean $\pm$ sem of n observations. Statistical analysis was performed using analysis of variance followed by Bonferroni adjustment, and p<0.05 was considered statistically significant.

## RESULTS

### Peroxynitrite inhibits I $\kappa$ B $\alpha$ phosphorylation and degradation, and NF- $\kappa$ B p65 nuclear translocation elicited by LPS or TNF $\alpha$

Phosphorylation of I $\kappa$ B $\alpha$ , which targets I $\kappa$ B $\alpha$  for degradation in the proteasome, allows inactive cytoplasmic NF- $\kappa$ B dimers to translocate into the nucleus (1,2). Stimulation of H9C2 cells with LPS or TNF $\alpha$  (Fig. 1A and B) triggered I $\kappa$ B $\alpha$  phosphorylation that was maximal at 10 min and rapidly declined thereafter, most likely due to its known proteasome-dependent degradation. Phosphorylation of I $\kappa$ B $\alpha$  was associated with I $\kappa$ B degradation, as evidenced by the marked reduction of I $\kappa$ B signal 30 min after LPS or TNF $\alpha$  (Fig. 1C and D). At 60 min post-stimulation, the I $\kappa$ B  $\alpha$  signal returned to baseline levels, consistent with I $\kappa$ B  $\alpha$  resynthesis triggered by the activation of NF- $\kappa$ B (26). Pretreatment of cells with 250  $\mu$ M PN for 20 min before LPS or TNF $\alpha$  prevented I $\kappa$ B  $\alpha$  to be phosphorylated, an effect associated to a complete inhibition of I $\kappa$ B $\alpha$  degradation. In contrast to the effects of PN, decomposed peroxynitrite (DP) did not exert any influence on I $\kappa$ B $\alpha$  (Fig. 1A–D). There were no significant changes in the expression levels of  $\alpha$ -tubulin between the different conditions, indicating equal loading of proteins (Fig. 1E and F).

Nuclear translocation of NF- $\kappa$ B p65 was evaluated by monitoring the levels of p65 (RelA) protein in nuclear extracts. Both LPS and TNF $\alpha$  induced RelA/p65 nuclear translocation, that occurred earlier with TNF $\alpha$  (10 min) than with LPS (30 min) and was maintained up to 1 hour after stimulation (Fig. 2A and B). In keeping with its effects on I $\kappa$ B $\alpha$  phosphorylation and degradation, PN (but not DP) abrogated the nuclear translocation of RelA/p65 elicited by LPS or TNF. The changes in nuclear RelA/p65 were clearly due to its nuclear translocation, but not to changes in the expression levels of RelA/p65, as shown by RelA immunoblots in cytoplasmic extracts (Fig. 2C and D).

### Peroxynitrite prevents NF- $\kappa$ B DNA binding in H9C2, HMEC-1 and EAhy 926 cells

There was no nuclear NF- $\kappa$ B DNA binding in quiescent H9C2 cells, as shown by bandshift experiments (Fig. 3). After 30 minutes of stimulation, LPS or TNF $\alpha$  strongly induced NF- $\kappa$ B DNA binding activity, which was abrogated by PN, but not DP (Fig. 3A and B). Concentration-response experiments indicated that PN already significantly inhibited DNA binding activity at a concentration of 10  $\mu$ M, with almost complete inhibition obtained at 100  $\mu$ M (Fig. 3C and D). To evaluate possible cell specificity of this effect of PN, NF- $\kappa$ B DNA binding was determined in two human endothelial cell lines, HMEC-1 and EAhy 926 stimulated with TNF $\alpha$ , IL-1 $\beta$ , or both. In HMEC-1 cells, NF- $\kappa$ B was activated by TNF $\alpha$  and IL-1 $\beta$ , and this was eliminated by PN (250 $\mu$ M), but not DP (Fig. 3E). EAhy 926 cells were found to be responsive to TNF $\alpha$ , but not IL-1 $\beta$ . NF- $\kappa$ B DNA binding elicited by TNF $\alpha$  was again markedly inhibited by PN pretreatment (Fig. 3F). Thus, the inhibitory effects of PN on NF- $\kappa$ B activation is observed independently from the stimulus (LPS, TNF $\alpha$ , IL-1 $\beta$ ) or the cell type studied.

### Peroxynitrite-dependent NF- $\kappa$ B inhibition depends on oxidation, but not nitration

The chemistry of PN encompasses both oxidation reactions and nitration of protein tyrosine residues (27). Distinction between these two kinds of reactions can be obtained with epicatechin, which acts as a potent inhibitor of tyrosine nitration elicited by PN (21). Stimulation with 250  $\mu$ M PN for 15 minutes induced a widespread nitration of cellular proteins in H9C2 cells, as evidenced both by western blotting and by immunocytochemical detection (Fig. 4A and B). Nitration was concentration-dependently inhibited by epicatechin, in accordance with previous data by other investigators (21), with complete inhibition obtained at 100  $\mu$ M and above. We thus used a concentration of 100  $\mu$ M epicatechin to evaluate the role of nitration on NF- $\kappa$ B inhibition by PN. As shown in Fig. 4C, the PN-dependent prevention of I $\kappa$ B $\alpha$  phosphorylation in response to LPS was not affected by epicatechin, indicating that PN



does not block NF- $\kappa$ B activation via nitration reactions and supporting that an oxidative type of chemistry is responsible for this effect. To further confirm that the inhibition of NF- $\kappa$ B by PN was due to oxidation, we assessed the possible effect of another oxidant species, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), on the process of NF- $\kappa$ B activation by LPS in H9C2 cells. As shown in Fig. 4D and E,  $\text{H}_2\text{O}_2$  prevented the DNA binding activity of NF- $\kappa$ B as did PN. However, in contrast to PN, the concentrations of  $\text{H}_2\text{O}_2$  required for this inhibition were higher (100  $\mu\text{M}$  and above). Also, the effect of  $\text{H}_2\text{O}_2$  could be fully reversed in the presence of catalase, whereas the latter did not affect the inhibition of NF- $\kappa$ B activation by PN (Fig. 4F and G). Finally, the PN-mediated inhibition of NF- $\kappa$ B-DNA binding triggered by LPS was completely prevented when cells were pretreated for 2h with 250 $\mu\text{M}$  of the superoxide dismutase (SOD) mimetics and antioxidant agent Mn(III)-tetrakis-(4-benzoic acid) porphyrin (MnTBAP) (Fig. 4H). Overall, these data indicate that PN-dependent inhibition of NF- $\kappa$ B occurs as a result of oxidative, but not nitrative processes.

### **Peroxynitrite inhibits IKK $\beta$ phosphorylation and IKK kinase activity, but activates the phosphorylation of NIK and IKK $\alpha$**

Activation of IKK represents the crucial step in the process of NF- $\kappa$ B activation (1). As shown in Fig. 5A and B, both LPS and TNF $\alpha$  enhanced IKK activity, as demonstrated by kinase assay experiments. Following LPS, IKK activation started at 10 min, reached a peak at 15–20 min and then steadily declined until 60 min. Upon stimulation with TNF $\alpha$ , the pattern was similar, although IKK activity was induced earlier than with LPS, starting as early as 5 min after TNF $\alpha$ , peaking at 5 to 10 min and disappearing thereafter. IKK activation was associated with an enhanced phosphorylation of both IKK $\alpha$  and IKK $\beta$  by LPS and TNF $\alpha$ , which was maximal at 20 min after LPS and 10 min after TNF $\alpha$  (Fig. 5A and B).

Due to the inhibition of NF- $\kappa$ B activation by PN, we assessed whether this effect could be related to the inhibition of IKK activation. The possible influence of PN was explored in cells stimulated with LPS for 20 min, or with TNF $\alpha$  for 10 min, as these time points corresponded to the peak activation of IKK by these stimuli. Fig. 5C (LPS) and 5D (TNF $\alpha$ ), unequivocally demonstrate that PN completely prevented the activation of IKK triggered by the two inflammatory stimuli.

Since IKK activation relies on the phosphorylation of two serine residues within the activation loops of IKK $\alpha$  and IKK $\beta$ (1), we examined the effects of PN on IKK phosphorylation. Both LPS (Fig. 5E) and TNF $\alpha$  (Fig. 5F) triggered the phosphorylation of IKK $\alpha$  and IKK $\beta$  after 20, respectively 10 min. When cells were pretreated with PN for 20 min, the phosphorylation of IKK $\beta$  was totally prevented, while PN did not exert any inhibition on the phosphorylation of IKK $\alpha$  (Fig. 5E and F, densitometric analysis, Fig. 5G and H). The effects of PN were not associated with significant changes in the expression levels of IKK $\alpha$ , IKK $\beta$  and  $\alpha$ -tubulin. Thus, PN prevents IKK activation by impairing specifically the phosphorylation of IKK $\beta$ .

We then sought to determine whether PN on its own has the ability to phosphorylate IKK $\alpha$ . As shown in figure 6A and B, The phosphorylation of IKK $\alpha$  in cells treated with LPS or TNF $\alpha$  was maintained for up to 2h only if they were pretreated with PN. Furthermore, PN alone, independently from any stimulation with LPS or TNF $\alpha$ , activated the phosphorylation of IKK $\alpha$ , an effect evident already after 10 min and further increasing at 15 and 20 min (Fig. 6C). Previous studies have shown that IKK $\alpha$  phosphorylation relies on the upstream activation of NIK (12,28), and we thus determined whether PN might activate this particular signaling cascade. As indicated in Fig. 6C, PN induced the phosphorylation of NIK, with a time-course parallel to that of IKK $\alpha$  phosphorylation.

## Peroxyntirite inhibits the transcription of NF- $\kappa$ B-dependent genes

In order to evaluate whether PN-dependent NF- $\kappa$ B inhibition might influence NF- $\kappa$ B-dependent gene transcription, we first addressed the influence of PN on NF- $\kappa$ B-luciferase reporter gene expression determined 4 hours after TNF $\alpha$  stimulation. While PN alone had no effect, TNF $\alpha$  induced more than a 20-fold increase in the expression of the transfected NF- $\kappa$ B-dependent luciferase gene (Fig. 7A). This increase was not modified by pretreatment of cells with DP, whereas PN induced an almost complete inhibition of luciferase expression. We then evaluated the concentration-dependence of this effect by pretreating cells with increasing concentrations (10–250  $\mu$ M) of PN and then TNF $\alpha$  or LPS for 4h. Fig. 7B and C show that PN at all concentrations significantly inhibited the TNF $\alpha$  and LPS-dependent luciferase expression. To make sure that these effects were not related to nonspecific cytotoxicity by PN, we assessed cell viability (MTT assay) in H9C2 cells 4 hours after a 20 min exposure to 250  $\mu$ M PN. As shown in Fig. 7D, PN only marginally affected cell viability, which indicates that the decrease in luciferase expression was indeed consecutive to NF- $\kappa$ B inhibition but not to cell death.

We also explored whether endogenous genes, which are known to be transcriptionally regulated by NF- $\kappa$ B, would be affected by PN. Both LPS and TNF $\alpha$  induced the transcription of the genes encoding inducible NO synthase (iNOS) (Fig. 8A and B) and TNF $\alpha$  (Fig. 8C and D), and this was not influenced when the cells were pretreated with DP. In contrast, PN completely abrogated the transcription of iNOS and TNF $\alpha$  in cells stimulated either with LPS or TNF $\alpha$ .

## DISCUSSION

Recent studies have indicated that PN is generated in large amounts in the myocardium during myocardial reperfusion injury (15), chronic heart failure (16), circulatory shock (29) and anthracycline cardiomyopathy (17). While PN has generally been considered as directly biotoxic towards cardiomyocytes, we now provide the novel evidence that PN also behaves as an important modulator of cell signal transduction at the level of NF- $\kappa$ B. A short exposure to PN exerted a marked inhibition towards I $\kappa$ B $\alpha$  phosphorylation and degradation, p65 nuclear accumulation and NF- $\kappa$ B DNA binding triggered by LPS or TNF $\alpha$  in H9C2 cardiomyocytes (Fig. 1–3). This inhibition was already significant at low concentrations of PN (10  $\mu$ M), with more than 80% inhibition obtained at 50  $\mu$ M of the oxidant. Similarly, PN inhibited NF- $\kappa$ B activation in 2 additional cell lines, HMEC-1 and EAhy 926 endothelial cells, stimulated with TNF $\alpha$  and IL-1 $\beta$  (Fig. 3E and F), which indicates that the inhibitory effect of PN on NF- $\kappa$ B is independent of type of cell and stimulus.

PN has been associated with two different types of chemistry, namely oxidation reactions and nitration of tyrosine residues. Our finding that epicatechin, which fully reversed the nitration induced by PN, did not affect the inhibition of NF- $\kappa$ B activation (Fig. 4A–C), indicates that oxidation rather than nitration was the relevant chemical reaction underlying this effect of PN. This conclusion is further supported by the findings that (a) another strong oxidant species, hydrogen peroxide, also inhibited NF- $\kappa$ B activation in H9C2 cells stimulated with LPS, although this effect was observed at concentrations higher than those of PN (Fig. 4D and E), and (b) the inhibition of NF- $\kappa$ B-DNA binding activity by PN was totally prevented by the SOD mimetics and antioxidant compound MnTBAP (Fig. 4H). These observations argue against the concept that NF- $\kappa$ B is a general sensor of cellular redox stress. Such redox sensitivity of NF- $\kappa$ B has been proposed on the basis that anti-oxidants can prevent NF- $\kappa$ B activation in response to cytokines and that NF- $\kappa$ B can be activated upon the direct exposure of cells to reactive oxygen species (7,30), but this issue is currently debated for a number of reasons. First, NF- $\kappa$ B can be activated by oxidant stress only in some, but not all cell types (8). Second, there are multiple mechanisms accounting for NF- $\kappa$ B activation in response to redox stress (8).



Oxidants have been shown to activate NF- $\kappa$ B either via IKK-dependent I $\kappa$ B $\alpha$  serine phosphorylation (31), or via tyrosine phosphorylation of I $\kappa$ B $\alpha$  at Tyr 42 (32). It has been claimed that this variability in the modes of action of oxidants does not support their role as universal NF- $\kappa$ B activators (8). Third, it has been demonstrated that endogenously produced reactive oxygen species do not mediate NF- $\kappa$ B activation in HeLa, HT-29, L-929 and Jurkat T-cells (33). Fourth, a recent report indicated that oxidant stress inhibited NF- $\kappa$ B activation by TNF $\alpha$  in alveolar RLE and C10 cells (34). Our data further challenge the issue of the redox responsiveness of NF- $\kappa$ B, by providing strong experimental evidence that a short episode of redox stress with PN potently inactivates consecutive activation of NF- $\kappa$ B by pro-inflammatory signals. Since PN is generated in many different pathological conditions, these findings may have important consequences to understand the ramification between PN formation and the regulation of inflammatory processes in vivo.

The key regulatory step in NF- $\kappa$ B activation is the activation of the upstream kinase IKK (1). The activation of IKK subunits relies in the phosphorylation of serine residues within their activation loops (Ser176 and 180 for IKK $\alpha$  and Ser177 and 181 for IKK $\beta$ ) (35). Here, we provide the first experimental evidence that PN prevents LPS and TNF $\alpha$ -dependent NF- $\kappa$ B activation through a complete blockade of IKK activity, due to inhibition of IKK $\beta$  phosphorylation (Fig. 5). These findings are consistent with previous data from the literature indicating that IKK $\beta$  represents a key target of various NF- $\kappa$ B inhibitors, including cyclopentone prostaglandins (36), arsenite (37) and NO (whose chemical reactivity is distinct from that of PN (14)).

Another striking observation of the present study was the opposite influence of PN towards the  $\alpha$  and  $\beta$  subunits of IKK. Whereas PN blocked LPS and TNF $\alpha$ -dependent IKK $\beta$  phosphorylation, it did not reduce, but instead promoted IKK $\alpha$  phosphorylation (Fig. 5 and 6). Such a contrasted influence of a single compound on IKK $\alpha$  and  $\beta$  has not been previously reported, but is consistent with the fact that phosphorylation of IKK $\alpha$  and IKK $\beta$  proceeds through distinct mechanisms. Whereas IKK $\beta$  phosphorylation critically depends on the regulatory subunit IKK $\gamma$ , IKK $\alpha$  can be directly phosphorylated by the upstream kinase NIK (1). Recent reports indicate that NIK/IKK $\alpha$  mediate an alternative, non canonical NF- $\kappa$ B pathway, in lymphoid cells in response to certain stimuli including LT $\beta$ R, BAFF and CD40, resulting into the processing of the NF- $\kappa$ B subunit p100 into p52 (9–11,38). Our finding that phosphorylation of IKK $\alpha$  by PN was associated with the simultaneous phosphorylation of NIK (Fig. 6C), suggests that redox stress mediated by PN may activate a similar pathway in cardiomyocytes. However, we did not find evidence of p100 processing into p52 in cells stimulated with PN (data not shown), implying that the downstream events triggered by PN-mediated phosphorylation of NIK and IKK $\alpha$  remain yet to be established.

Beside its function in non canonical NF- $\kappa$ B activation, a novel role of IKK $\alpha$  in the canonical pathway of NF- $\kappa$ B activation has been recently identified by Lawrence and coworkers (39). In this study, mice expressing an inactivatable variant IKK $\alpha$  (*ikka*<sup>AA/AA</sup>) showed a markedly increased inflammatory response following LPS administration, characterized by an increased expression of NF- $\kappa$ B-target genes in tissues (lung and liver). Furthermore, LPS-stimulated *ikka*<sup>AA/AA</sup> macrophages disclosed a markedly prolonged NF- $\kappa$ B-DNA binding activity relative to wild-type cells, related to a reduced degradation of the NF- $\kappa$ B subunits RelA (p65) and c-Rel in *ikka*<sup>AA/AA</sup> macrophages (39). Overall, the results of this study clearly identified a previously unrecognized function of IKK $\alpha$  as a negative regulator of NF- $\kappa$ B activation, indispensable for the resolution of inflammation. Thus, IKK $\alpha$  and IKK $\beta$  play opposite (down-versus upregulation), yet complimentary roles in inflammation. Our results that PN-mediated inhibition of NF- $\kappa$ B activation is associated with an opposite regulation of IKK $\alpha$  (activation) and IKK $\beta$  (inhibition) is therefore consistent with the distinct functions of these subunits reported in the study by Lawrence et al.

In keeping with the findings that PN blocked IKK $\beta$ -dependent NF- $\kappa$ B activation, we found that it also markedly reduced the transcription of NF- $\kappa$ B dependent genes in cardiomyocytes. Using a luciferase reporter assay, we found a marked reduction of luciferase activity in cells stimulated with TNF $\alpha$  or LPS after PN pretreatment (Fig. 7A–C). Also, PN completely blocked the transcription of the endogenous inflammatory genes encoding iNOS and TNF $\alpha$  (Fig. 8). Importantly, both TNF $\alpha$  and NO generated from iNOS have been proposed as potential myocardial depressant effectors in pathologies such as septic shock (40) and chronic heart failure (41), where enhanced myocardial formation of PN has also been documented. Our data therefore suggest that PN generation in vivo might unexpectedly down-regulate the expression of pro-inflammatory mediators and cardiodepressant molecules, providing a counterregulatory mechanism to prevent overt inflammation and myocardial failure in various pathological conditions.

In summary, our data show that PN, acting through oxidative but not nitrative chemistry, is a potent inhibitor of canonical NF- $\kappa$ B activation triggered by inflammatory cytokines or LPS, as proposed in the diagrammatic scheme presented in figure 9. This effect is related to the blockade of IKK $\beta$  phosphorylation, resulting in the inactivation of the kinase and downstream signaling. The inhibition of IKK $\beta$  dependent NF- $\kappa$ B activation by PN translates into a complete inhibition of the transcription of NF- $\kappa$ B dependent genes. Furthermore, PN simultaneously activates NIK and IKK $\alpha$  phosphorylation, suggesting its potential role in an alternative pathway of NF- $\kappa$ B activation, whose cellular consequences remain to be clarified. These findings offer new perspectives in understanding the relationships between oxidant stress and inflammation.

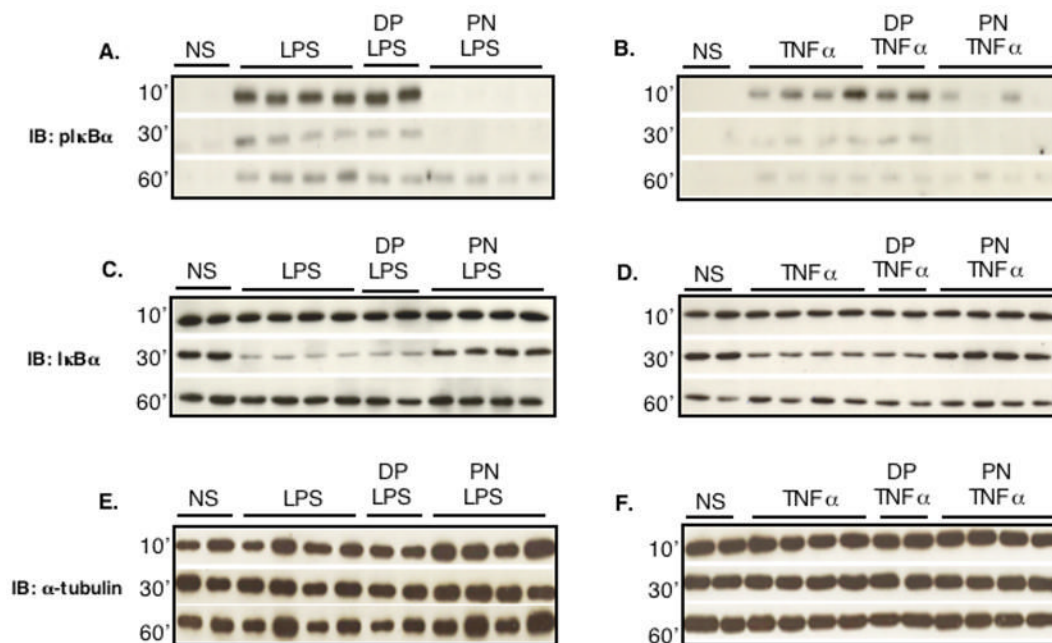
#### Acknowledgements

Supported by grants from the Swiss National Fund for Scientific Research (3100-65005.01 and PP00B-68882/1) to LL.

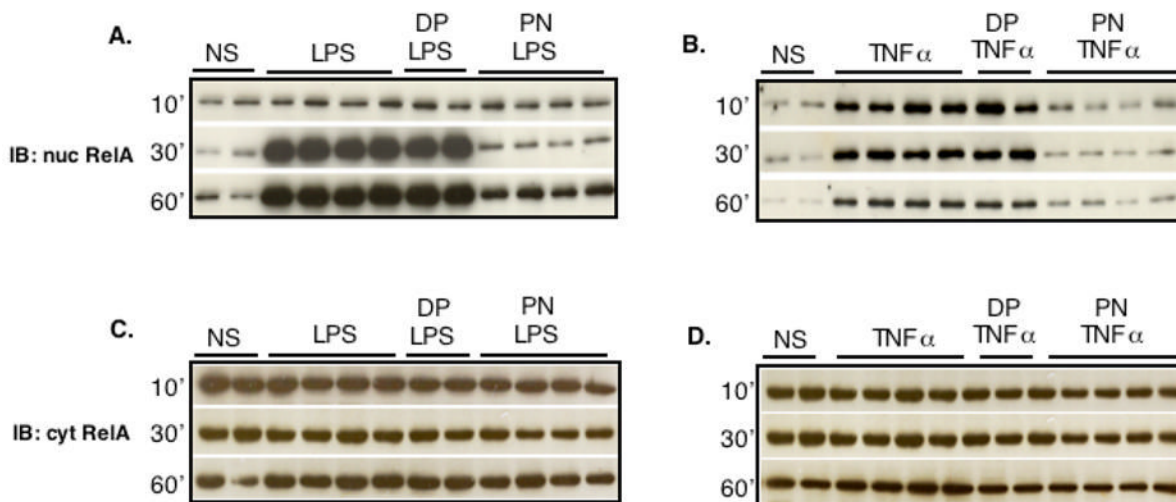
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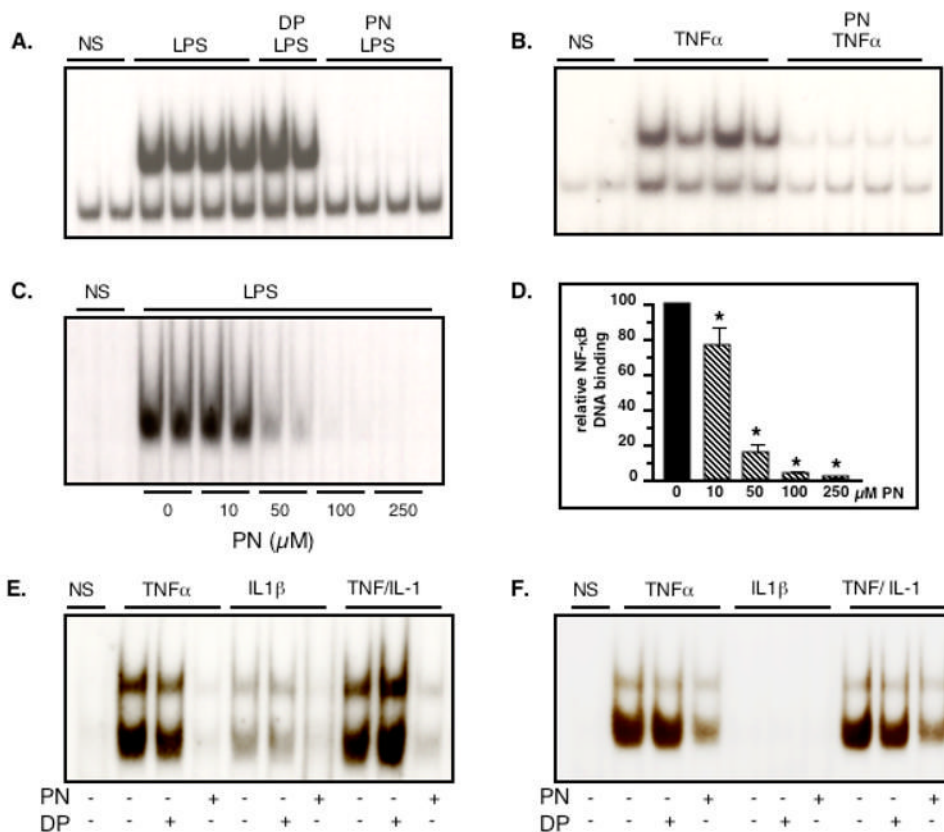
**Fig. 1.** Peroxynitrite inhibits I $\kappa$ B $\alpha$  phosphorylation and degradation triggered by inflammatory stimuli in H9C2 cells. H9C2 cells were either not stimulated (NS) or stimulated with LPS (1 $\mu$ g/ml, A, C, E) or TNF $\alpha$  (20 ng/ml, B, D, F) after pretreatment with either decomposed peroxynitrite (DP) or authentic peroxynitrite (PN, 250  $\mu$ M) for 20 min. Both LPS and TNF $\alpha$  induced I $\kappa$ B $\alpha$  phosphorylation (A, B) and degradation (C, D), which were prevented by PN but not DP. Expression levels of  $\alpha$ -tubulin (E, F) are shown as loading controls.



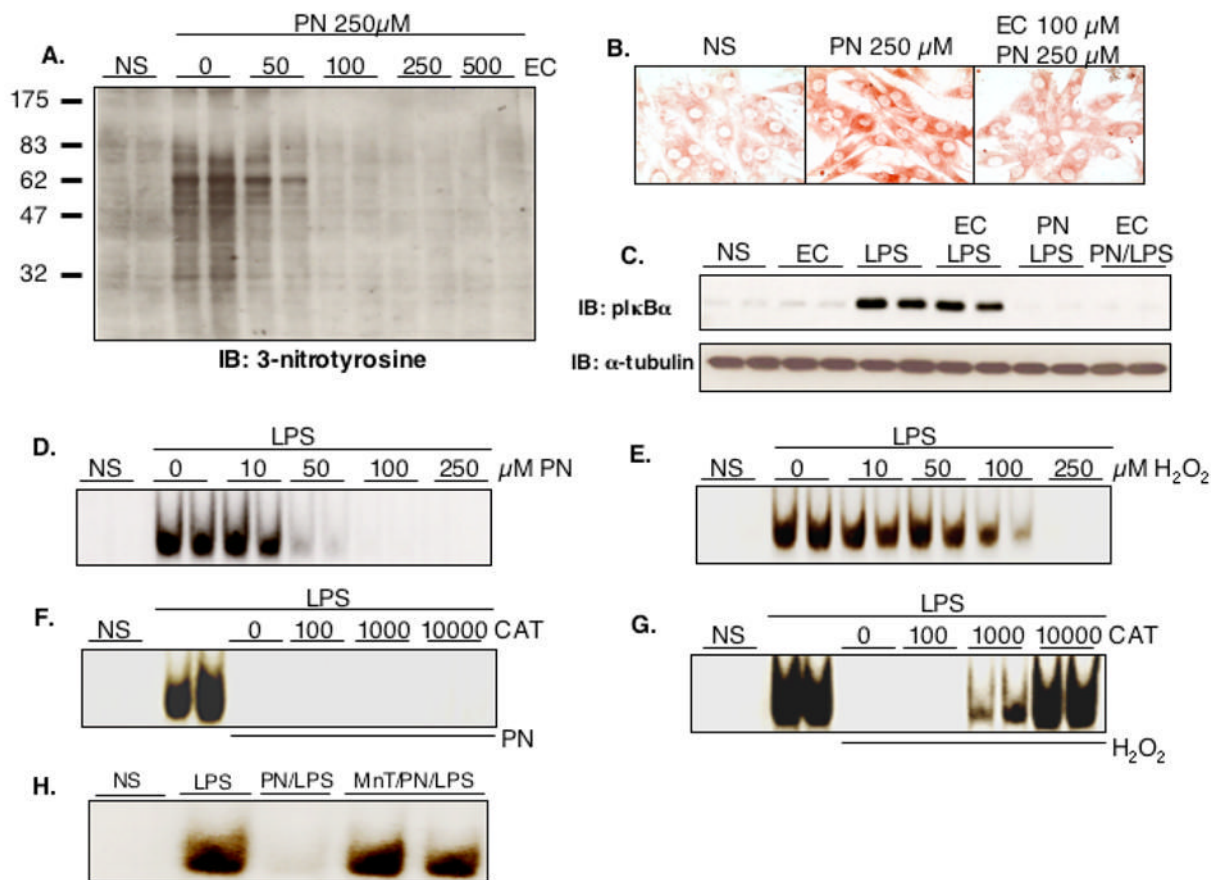
**Fig. 2.**

Peroxyntirite prevents the nuclear translocation of the p65 (RelA) subunit of NF- $\kappa$ B induced by LPS or TNF $\alpha$ . H9C2 cells were left unstimulated (NS) or were stimulated with LPS (1 $\mu$ g/ml, A, C) or TNF $\alpha$  (20 ng/ml, B, D) after a 20 min pretreatment with 250  $\mu$ M peroxyntirite (PN) or decomposed peroxyntirite (DP) for 20 min. Nuclear RelA accumulation was markedly increased following LPS or TNF $\alpha$  (A, B), with no changes in the levels of cytoplasmic RelA (C, D). PN, but not DP, prevented RelA nuclear translocation.



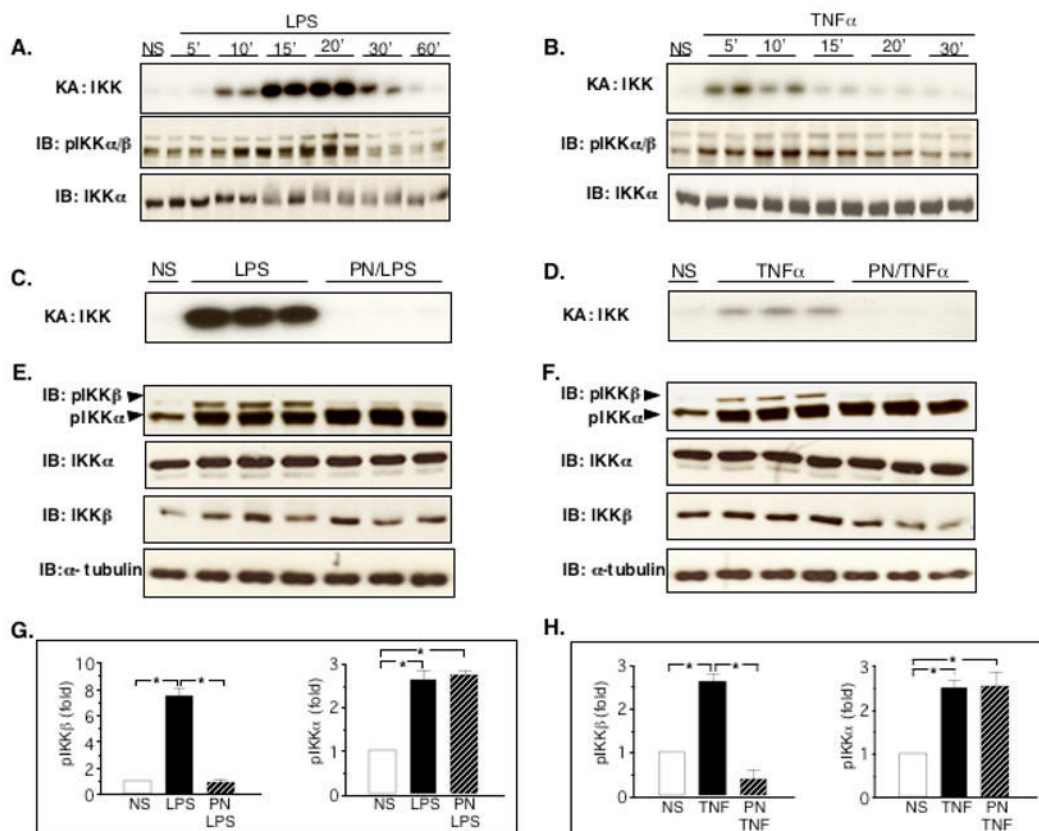


**Fig. 3.** Peroxynitrite inhibits NF- $\kappa$ B DNA binding activity triggered by inflammatory stimuli in H9C2, HMEC-1 and EAhy 926 cells. H9C2 cells were not stimulated (NS) or treated with LPS (1 $\mu$ g/ml, A) or TNF $\alpha$  (20 ng/ml, B). Both LPS and TNF triggered NF- $\kappa$ B DNA binding, an effect suppressed by pretreatment with peroxynitrite (PN, 250  $\mu$ M, 20 min), but not decomposed PN (DP). The inhibitory effect of PN on LPS-mediated NF- $\kappa$ B DNA binding was concentration-dependent (C, D). PN, but not DP, also inhibited NF- $\kappa$ B DNA binding in HMEC-1 cells (E) and EAhy 926 cells (F) stimulated with TNF $\alpha$  (20 ng/ml), IL1 $\beta$  (1 ng/ml) or both. Bar graph shows mean $\pm$ sem of at least 4 observations. \*  $p < 0.05$  (ANOVA followed by Bonferroni adjustments)

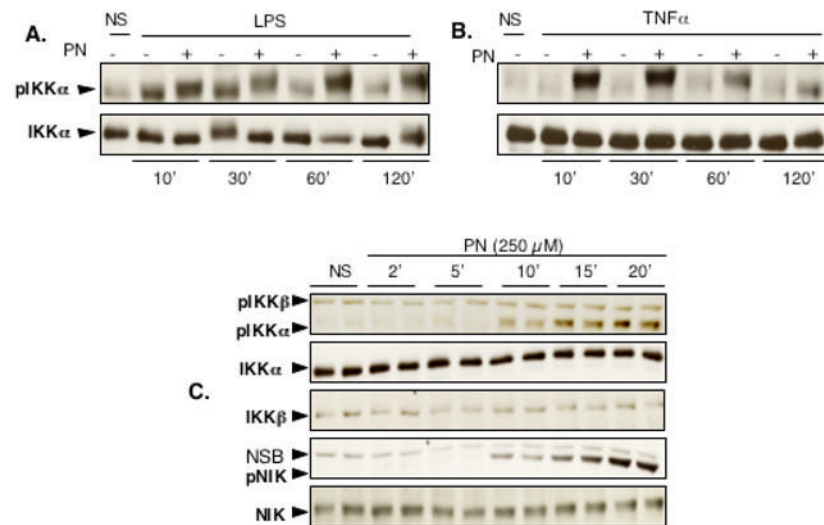


**Fig. 4.**

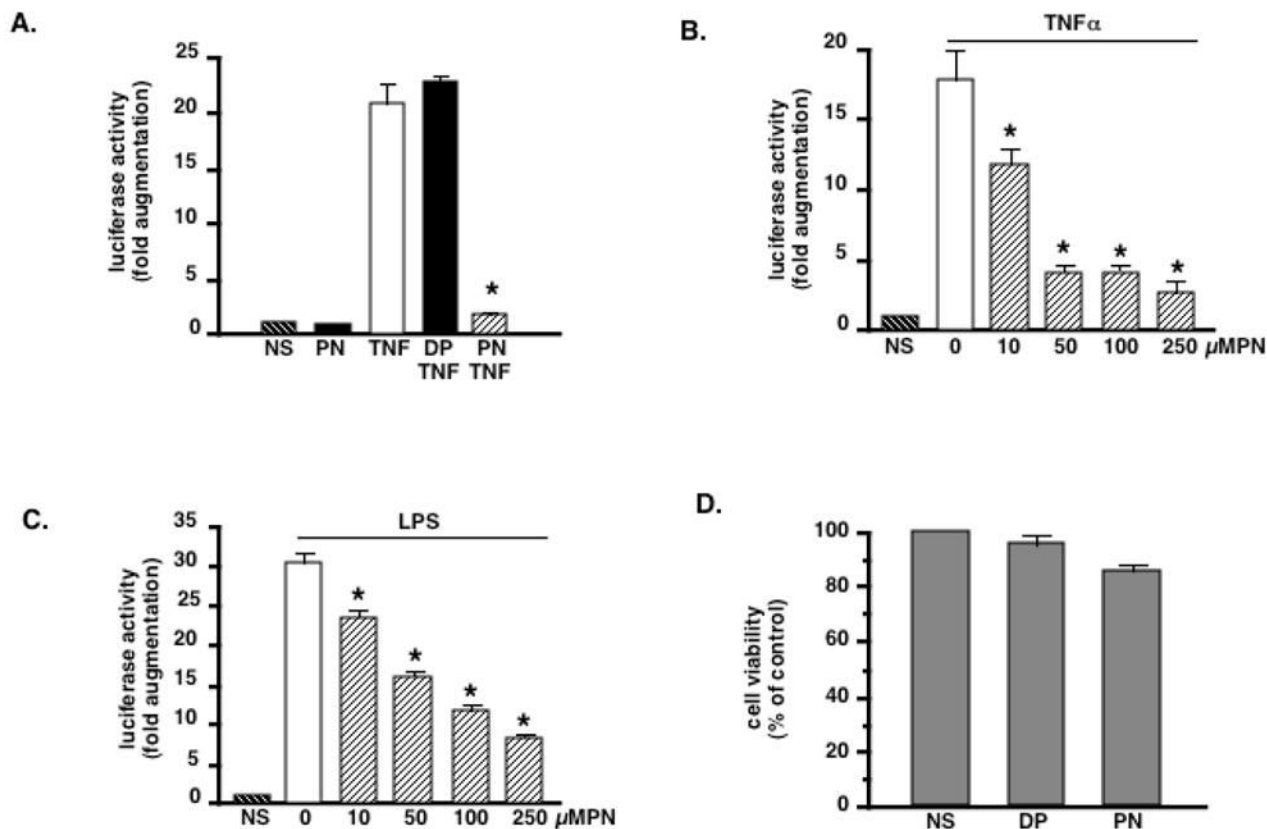
Peroxynitrite inhibits NF- $\kappa$ B independently from nitration reactions. Treatment of H9C2 cells for 20 min with 250  $\mu$ M peroxynitrite (PN) induced nitration of cellular proteins, as indicated by the formation of 3-nitrotyrosine, which was abrogated by pretreatment with epicatechin (EC) at concentrations of 100  $\mu$ M and above (A, western, B, immunocytochemistry). Although 100  $\mu$ M EC efficiently prevented tyrosine nitration, it did not influence the PN dependent inhibition of NF- $\kappa$ B activation in cells stimulated with LPS, as assessed by I $\kappa$ B $\alpha$  phosphorylation (C). Expression levels of  $\alpha$ -tubulin are shown as a loading control. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), another oxidant species, was also able to inhibit NF- $\kappa$ B, as shown by band shift experiments, although this effect required higher concentrations than PN (D, E). The inhibitory effect of H<sub>2</sub>O<sub>2</sub> on NF- $\kappa$ B DNA binding was reversed in the presence of 100–10000 Units catalase (CAT), whereas the effect of PN was not influenced by catalase (F, G). Finally, the prevention of NF- $\kappa$ B-DNA binding activation produced by PN was reversed after 2h pretreatment with 250  $\mu$ M of the SOD mimetics and antioxidant MnTBAP (H). NS: Not Stimulated. MnT: MnTBAP.

**Fig. 5.**

Peroxyntirite inhibits IKK kinase activity by preventing specifically the phosphorylation of IKK $\beta$  in H9C2 cells. A and B: IKK kinase assay (KA) in cells treated with LPS or TNF $\alpha$ . LPS strongly activated IKK kinase from 10 to 30 minutes (A), whereas the effects of TNF $\alpha$  were most pronounced at 5 and 10 minutes (B). The activation of IKK was associated with an increased phosphorylation of IKK $\alpha$  and IKK $\beta$  by LPS and TNF $\alpha$ . IKK $\alpha$  levels are shown as an internal control. C and D: IKK kinase activity in cells pretreated with peroxynitrite (PN, 250  $\mu$ M, 20 min) and stimulated with LPS (C, 20 min) or TNF $\alpha$  (D, 10 min). PN abrogated IKK activity triggered by LPS or TNF $\alpha$ . E and F: Phosphorylation of IKK $\alpha$  and  $\beta$  was induced both by LPS (20 min, E) and TNF $\alpha$  (10 min, F). PN completely prevented the phosphorylation of IKK $\beta$  triggered by LPS and TNF $\alpha$ , whereas it did not reduce IKK $\alpha$  phosphorylation in response to LPS and TNF $\alpha$ . No significant changes in the levels of IKK $\alpha$ , IKK $\beta$  and  $\alpha$ -tubulin were found between the different conditions. G and H: densitometric analysis of phospho-IKK western blots showing mean  $\pm$  sem of 3 observations. NS: Not Stimulated. \*  $p < 0.05$ , ANOVA followed by Bonferroni adjustment.

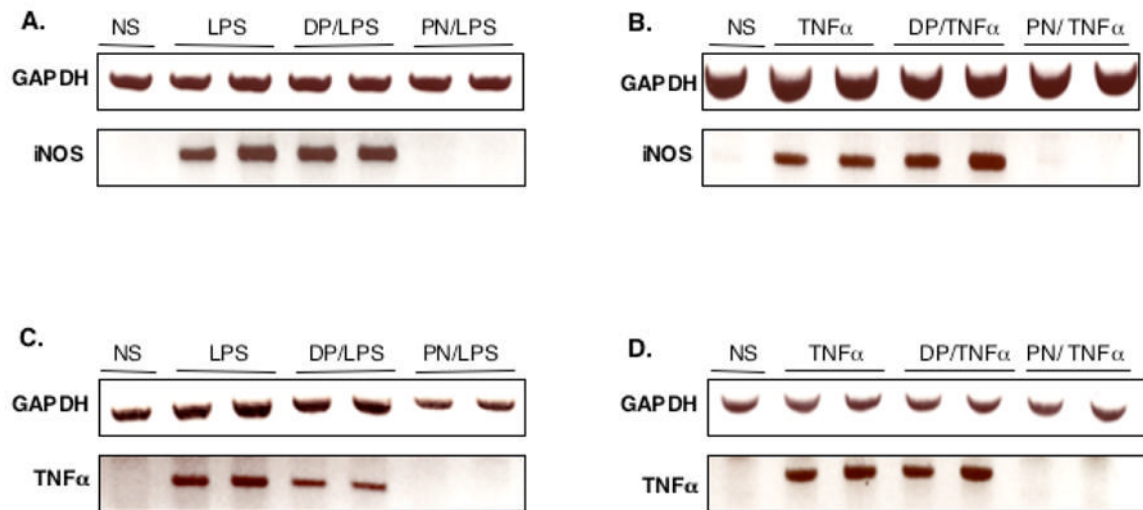


**Fig. 6.** PN activates the phosphorylation of IKK $\alpha$  and NIK. IKK $\alpha$  phosphorylation was monitored in cells stimulated with LPS (A) or TNF $\alpha$  (B) for 10 to 120 min, in the absence or presence of peroxynitrite (PN). In the presence of PN, there was a strong phosphorylation of IKK $\alpha$ , maintained for up to 120 min after LPS or TNF $\alpha$ , with no change in total IKK $\alpha$ . C: PN alone strongly activated the phosphorylation of IKK $\alpha$  and NIK, an effect starting after 10 min. NS: Not Stimulated. NSB: non specific band

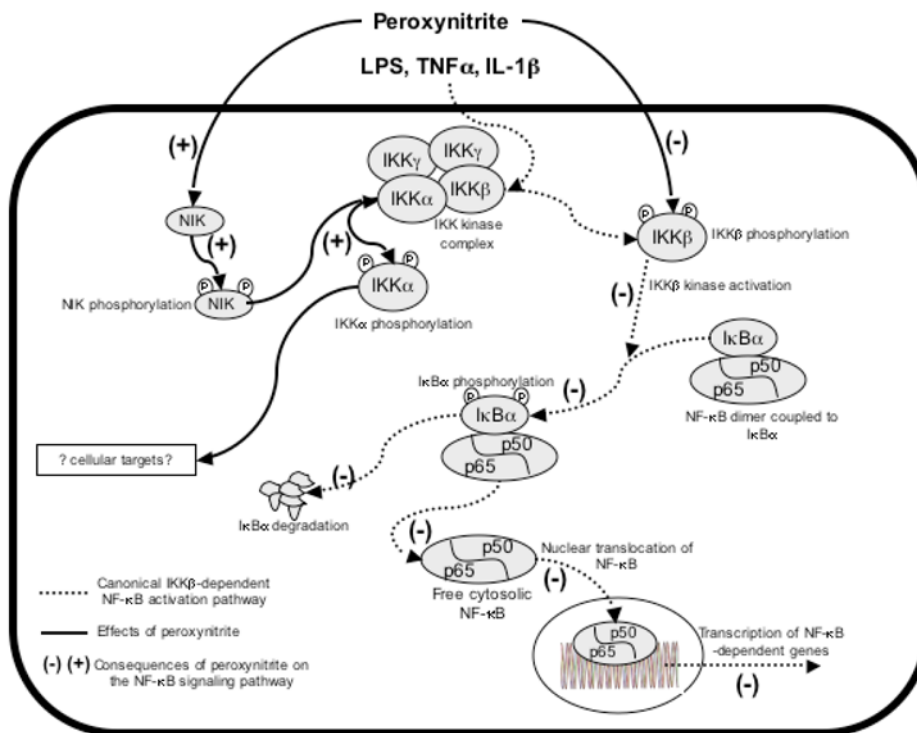


**Fig. 7.** PN inhibits the activity of a NF- $\kappa$ B-luciferase gene reporter activated by LPS or TNF $\alpha$ . Cells were either not stimulated (NS), or stimulated with peroxynitrite alone (PN, 250 $\mu$ M, 20 min), or TNF $\alpha$  (20 ng/ml) following pretreatment for 20 min with decomposed peroxynitrite (DP) or PN. Luciferase activity was measured after 4 h (A). PN alone had no effect, while TNF $\alpha$  induced a 20-fold increase in luciferase activity, an effect unchanged by DP, but significantly prevented by PN. B and C: PN-dependent inhibition of luciferase activity triggered by TNF $\alpha$  (B) or LPS (C) is concentration-dependent. D. The effects of PN are not related to non specific cytotoxicity. Cells were treated with PN (250  $\mu$ M, 20 min) or DP (20 min). Cell viability was assessed 4 h later by the MTT assay. PN only marginally affected cell survival (86% viability at 4h). Bar graphs are means  $\pm$  sem of at least 4 measurements. \*  $p < 0.05$  vs TNF or LPS alone (ANOVA followed by Bonferroni adjustments)





**Fig. 8.** PN inhibits the transcription of endogenous NF- $\kappa$ B-dependent genes. The transcription of inducible nitric oxide synthase (iNOS) and TNF $\alpha$  mRNA was evaluated in response to LPS (A, C) or TNF $\alpha$  (B, D) stimulation for 2h. LPS and TNF induced iNOS and TNF $\alpha$  gene transcription, an effect abrogated by pretreatment with peroxynitrite (PN, 250  $\mu$ M, 20 min), but not decomposed PN (DP, 20 min). NS: Not Stimulated.

**Fig. 9.**

A model for peroxynitrite-mediated interference with the NF- $\kappa$ B pathway. Immune stimuli activate the canonical NF- $\kappa$ B pathway, by inducing phosphorylation and activation of IKK $\beta$ , in a process regulated by IKK $\gamma$ . In turn, IKK $\beta$  phosphorylates I $\kappa$ B $\alpha$ , leading to its proteasomal degradation. NF- $\kappa$ B dimers (mostly p50/p65) then translocate into the nucleus, bind to  $\kappa$ B consensus sequences, and activate the transcription of target genes. In the presence of peroxynitrite, IKK $\beta$  phosphorylation is impaired, thereby blocking IKK $\beta$  activation and downstream signaling (as indicated by the (-) signs). At the same time, peroxynitrite activates the phosphorylation of IKK $\alpha$ , most probably via the phosphorylation and activation of the upstream kinase NIK. The cellular targets of IKK $\alpha$  in this model remain to be defined.

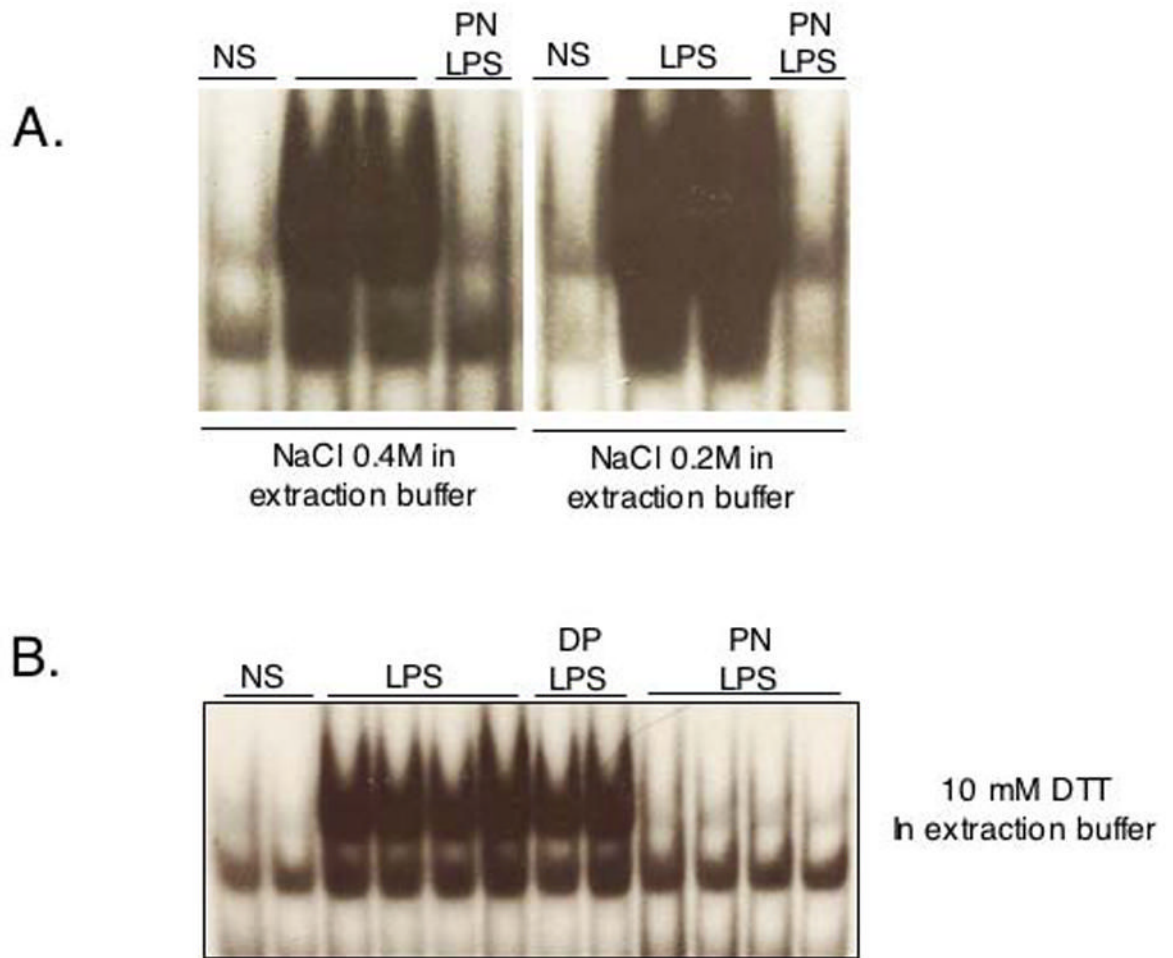


Fig. 10.