Activation of transcription factor NF-κB by phagocytic stimuli in human neutrophils

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Abstract Phagocytosis represents an important physiological trigger for the inducible expression of several genes in human neutrophils. Here, we report that a DNA-binding activity primarily consisting of the classical NF-κB heterodimer, p50/RelA, is induced in phagocytosing neutrophils. Under these conditions, NF-κB activation was found to be a rapid and transient response, reaching a maximum by 10–15 min, and returning to near-basal levels by 30 min. In neutrophils undergoing the phagocytosis of opsonized yeasts, the onset of NF-κB activation was paralleled by a decline in immunoreactive IκB-α protein levels, and the cellular IκB-α pool was replenished by 30 min, in agreement with our gel shift data. We conclude that NF-κB activation could constitute one of the mechanisms whereby the expression of κB-responsive genes is enhanced in phagocytosing neutrophils. To our knowledge, this represents the first demonstration that phagocytic stimuli can induce NF-κB activation in human neutrophils.

Key words: Neutrophil; NF-κB; Phagocytosis

1. Introduction

Since its initial discovery 10 years ago [1], the NF-κB/Rel family of transcription factors has emerged as a key component of various biological processes, especially within the context of inflammatory and immune responses, but also with respect to cell proliferation/differentiation and viral replication (reviewed in references [2] and [3]). In resting cells, NF-κB dimers are sequestered in the cytoplasm through an association with inhibitor molecules, collectively referred to as IκB proteins. Amongst the latter, the best studied example is IκB-α, which becomes rapidly phosphorylated upon cell activation — an event which is thought to target the molecule for its subsequent degradation [4]. This in turn causes the release of NF-κB dimers which translocate into to the nucleus where they can bind to specific enhancer sequences in the promoter region of target genes.

In keeping with its central involvement in host defence processes, the NF-κB pathway can be activated by a wide array of stimuli in various cells of the immune system such as lymphocytes, monocytes, and macrophages, amongst others [2,3]. Likewise, we recently showed that peripheral blood neutrophils respond to LPS[1], pro-inflammatory cytokines, and certain chemoattractants with a nuclear accumulation of NF-κB/Rel proteins, which is paralleled by the onset of nuclear NF-κB DNA-binding activity [5]. These observations are consistent with the known ability of neutrophils to accumulate mRNA transcripts encoding κB-dependent cytokines and chemokines (such as TNFα, IL-1β and IL-8) in response to the same stimuli (extensively reviewed in reference [6]). Interestingly, neutrophils have also been reported to rapidly express high levels of mRNA transcripts encoding TNFα and IL-8 in response to another class of stimuli, i.e. phagocytic particles [7–9]. In this regard, yeast particles were even shown to represent a more potent stimulus than LPS for the early induction of IL-8 and TNFα mRNA accumulation (as well as secretion) in neutrophils [7–9]. These considerations therefore prompted us to determine whether phagocytosis might trigger NF-κB activation in human neutrophils. We now report that NF-κB is rapidly and transiently induced in neutrophils undergoing the phagocytosis of heat-killed yeast particles. This represents the first demonstration that phagocytic stimuli can induce NF-κB activation in this cell type.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit antisera to human c-Rel (#1136, raised against an internal sequence downstream from the RHD), p65/RelA (#1207, against the N-terminal region), and p50/NFκB1 (#1141, against the N-terminal region) were a generous gift from Dr. N.R. Rice (NCI-Frederick Cancer Research and Development Center, Frederick, MD). The specificity of these antisera has already been extensively characterized [10,11]. The antibody to IκB-α (sc-371) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). An oligonucleotide containing tandemly repeated NF-κB sites identical to those of the HIV promoter (5′-gatcaGGGACTTTCCgctgGGGACTTTCC-3′; the NF-κB binding sequence is indicated in capital letters) was kindly provided by Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). Recombinant human (rh) TNFα was obtained from Bachem Inc. (Hannover, Germany). Ficoll-Paque, T4 polynucleotide kinase, poly(dI-dC), and Sephadex G25 spin columns were purchased from Pharmacia (Uppsala, Sweden); [γ-^{32}P]ATP was from ICN (Cleveland, OH). RPMI-1640 was from GIBCO/BRL (Gaithersburg, MD), and low-endotoxin FCS (< 6 pg/ml) from Hyclone (Logan, UT). Polystyrene tubes for cell culture were from Greiner (Nurtingen, Germany). Aprotinin, acetylated BSA, diisopropyl fluorophosphate (DFP), leupeptin, LPS, pepstatin A, and phenylmethanesulphonyl fluoride (PMSF) were all from the Sigma Chemical Co. (St. Louis, MO). All other reagents were molecular biology grade, and all buffers and solutions were prepared using pyrogen-free clinical grade water.

2.2. Cell isolation and culture

Neutrophils were isolated from the peripheral blood of healthy donors under endotoxin-free conditions by a modification of the method of Bøyum [12], as described earlier [7]. As determined by non-specific esterase cytochemistry, the final neutrophil suspensions consistently contained fewer than 0.5% monocytes, and neutrophil viability exceeded 98% after up to 3 h in culture, as determined by Trypan Blue exclusion. Purified neutrophils were resuspended in RPMI-1640 supplemented with 5% low-endotoxin FCS, at a final concentration of 5×10^6 cells/ml, and cultured in tubes at 37°C with frequent agitation. After being allowed to equilibrate for 15 min at 37°C, neutrophils were stimulated with LPS (1 μg/ml), TNFα (100 U/

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ml), or with heat-killed yeasts (*S. cerevisiae*, at a particle/cell ratio of 2:1) opsonized with IgG [13].

2.3. Electrophoretic mobility shift assays (EMSA)

Neutrophils were cultured in the presence or absence of the stimuli for the indicated times; incubations were stopped by transferring aliquots of the cell suspensions into pre-cooled tubes containing equivalent volumes of ice-cold RPMI-1640 supplemented with DFP (2 mM, final concentration) prior to centrifugation at $2000\times g$ for 2 min at 4°C. The cells were resuspended in ice-cold relaxation buffer (10 mM PIPES, pH 7.30, 30 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 0.5 mM DTT) containing an anti-protease cocktail (2 mM DFP, 1 mM PMSF, 1 mM AEBSF, and 10 μg/ml each of aprotinin, leupeptin and pepstatin A, final concentrations). Following a 10-min incubation on ice, cells were disrupted by nitrogen cavitation, using a modification of a previously published procedure [14] which we described elsewhere [5]. Whole-cell extracts were subsequently prepared and analyzed in EMSA exactly as described previously [5].

2.4. Denaturing electrophoreses and immunoblots

Whole-cell extracts were electrophoresed on 18% gels prepared according to the method of Thomas and Kornberg [15]. Proteins were transferred onto nitrocellulose membranes in a Transblot semidry transfer cell (BioRad), and the membranes were blocked in TBS–Tween containing 1.5% low-fat skimmed milk, as described [5]. Membranes were subsequently exposed to a 1:300 dilution of commercial anti-IκB-α antibody (sc-371) for 60 min at 37°C, washed 3 times with 150 ml of TBS, and incubated in TBS with a horseradish peroxidase-linked donkey anti-rabbit antibody, added to a final dilution of 1:10000, for 45 min at 37°C. After three washes, the signal was revealed with the ECL reagent, according to the manufacturer's instructions.

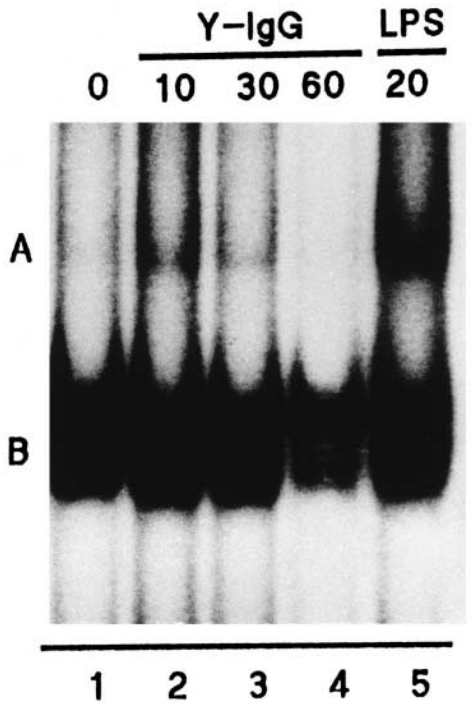

Fig. 1. Induction of NF-κB DNA-binding activity in human neutrophils undergoing phagocytosis of yeast particles. Neutrophils were cultured for the indicated times (in minutes) in the presence of IgG-opsonized yeast particles (Y-IgG), at a particle/cell ratio of 2:1. As a positive control, neutrophils were also stimulated with 1 μg/ml LPS. Whole-cell extracts were then prepared and analyzed in EMSA using a consensus NF-κB oligonucleotide probe. The amount of extract used in the binding reactions was 22 μg of protein (representing ≈730000 cell equivalents). Both DNA-binding activities (A,B) present in the neutrophil extracts have been previously shown to display specificity in their interaction with our NF-κB probe [5]. This experiment is representative of three.

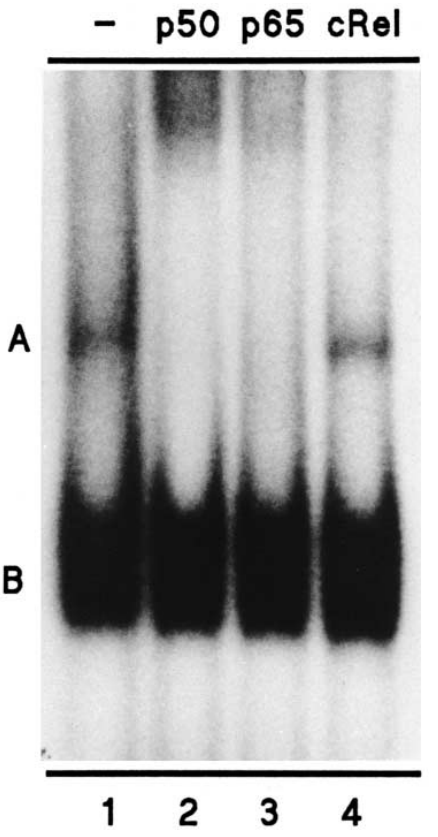

Fig. 2. Characterization of the NF-κB DNA-binding activities detected in phagocytosing neutrophils. Whole-cell extracts from neutrophils cultured for 10 min in the presence of Y-IgG were incubated in the absence (–) or in the presence of antisera raised against p65/RelA, p50, or c-Rel, prior to the addition of labeled NF-κB probe and subsequent EMSA analysis. The amount of extract used in the binding reactions was 30 μg of protein. This experiment is representative of two.

3. Results

To determine whether NF-κB is activated in phagocytosing neutrophils, the cells were cultured in the presence of IgG-opsonized yeast particles (hereafter referred to as Y-IgG) for varying lengths of time, prior to whole-cell extract preparation and subsequent EMSA analysis. As a positive control, neutrophils were also stimulated with 1 μg/ml LPS or 100 U/ml TNFα, two stimuli previously shown to induce NF-κB in these cells [5]. As shown in Fig. 1, whole-cell extracts from unstimulated neutrophils contained a weak constitutive NF-κB DNA-binding activity (complex A), as well as a faster-migrating doublet (complex B) which strongly bound our NF-κB probe. By comparison, phagocytosis of Y-IgG (lanes 2–4) led to a substantial increase in the intensity of complex A; this effect was transient, being maximal at 10–15 min of stimulation, and decreasing thereafter. In three independent experiments, the effect of Y-IgG stimulation towards complex A was of lesser magnitude than that exerted by LPS or TNFα. Finally, in contrast to complex A, the detection of complex B was largely unaffected by any of the agonists used, in agreement with previously reported observations [5].

We next sought to determine the subunit composition of the NF-κB complexes detected in extracts from activated neutrophils. To this end, supershift experiments were performed,

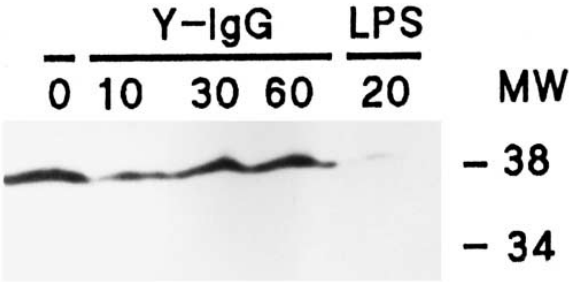

Fig. 3. Inducible degradation of IκB-α in phagocytosing neutrophils. Neutrophils were cultured for the indicated times (in minutes) in the presence Y-IgG or LPS, as described in the legend to Fig. 1. The resulting whole-cell extracts were then processed for electrophoresis (1.5×10^6 cell equivalents per well) and immunoblotting using an anti-IκB-α antibody (sc 371). For comparative purposes, the immunoblot shown in this figure was performed using extracts from the same experiment as the one depicted in Fig. 1. This experiment is representative of three. MW, molecular weight markers (in kDa).

using specific antisera raised against individual NF-κB/Rel proteins. As shown in Fig. 2, antisera to either RelA or p50 completely supershifted the inducible complex A present in extracts from Y-IgG-treated neutrophils (Fig. 2, lanes 2 and 3), while an antiserum to c-Rel only weakly affected the detection of this DNA-binding activity (Fig. 2, lane 4). It therefore appears that the inducible complex A principally consists of p50/RelA dimers, and that it may additionally contain small amounts of c-Rel-containing complexes. The similarly migrating NF-κB complex present in extracts from either LPS- or TNF-treated neutrophils was identically affected by the same antisera [5], indicating that its composition is likely to be the same. By contrast, complex B was unaffected by any of the antisera, as previously observed [5].

In a final series of experiments, we examined whether the induction of NF-κB DNA-binding activity by phagocytic stimuli might be paralleled by a loss of IκB-α, as previously observed in the case of TNFα or LPS [5]. For this purpose, whole-cell extracts from Y-IgG-stimulated neutrophils were analyzed by immunoblot. Fig. 3 shows that relative to unstimulated neutrophils (Fig. 3, lane 1), immunoreactive IκB-α levels had substantially decreased by 10 min in Y-IgG-stimulated cells (Fig. 3, lane 2); under these conditions, the cellular pool of IκB-α was fully replenished by 30 min (Fig. 3, lane 3). By comparison, immunoreactive IκB-α was almost entirely lost in neutrophils stimulated with LPS for 20 min, as previously observed [5]. Collectively, these observations are in good agreement with our gel shift data, insofar as the relative ability of the various stimuli to elicit NF-κB activation is mirrored by their respective ability to induce a transient decline in IκB-α levels.

4. Discussion

The NF-κB pathway is known to become activated under such diverse conditions as oxidative stress, viral challenge and ultraviolet irradiation, as well as in response to soluble stimuli such as inflammatory cytokines and LPS [3]. In the current study, we report that yet another class of stimuli, opsonized phagocytic particles, have the ability to activate NF-κB. In phagocytosing neutrophils, NF-κB activation was found to be a rapid and transient phenomenon, as it was already maximal by 10–15 min, and had returned to near-basal levels by 30 min. Under these conditions, maximal NF-κB activation was paralleled by a decline in immunoreactive IκB-α, which was however less marked than that observed following LPS or TNFα challenge, consistent with the ability of the latter stimuli to activate NF-κB to a greater extent than Y-IgG. Nevertheless, it cannot be excluded that a rapid accumulation of newly synthesized IκB-α might lead to an underestimation of the ability of Y-IgG to induce IκB-α degradation. In keeping with this interpretation is that cellular IκB-α levels were already replenished within 30 min in Y-IgG-treated neutrophils. Finally, the fast kinetics of IκB-α re-synthesis might account for the relatively brief time interval over which NF-κB activation takes place in phagocytosing neutrophils.

Although the sequence of events that link particle ingestion to NF-κB activation remains to be established, several clues point to the involvement of distinct cell surface receptors in this process. For instance, CR3 (CD11b/CD18) has been reported to function as a receptor for unopsonized zymosan (a yeast cell wall constituent) in human neutrophils [16], based on its ability to specifically recognize the β-glucan moiety of zymosan [17]. Consistent with these observations is that the ingestion of unopsonized zymosan by neutrophils appears to be primarily dependent upon its β-glucan content [18]. Interestingly, stimulation of CR3 using agonistic monoclonal antibodies was recently shown to induce the formation of a nuclear NF-κB DNA-binding activity consisting of p50/RelA in peripheral blood monocytes, as well as in HIV-infected monocytic cell lines [19]. Taken together, the above considerations raise the possibility that the effect of yeast phagocytosis towards NF-κB activation might be mediated (at least in part) by CR3 in neutrophils. On a related note, the phagocytosis of zymosan by neutrophils has been observed to promote microtubule assembly within 3 minutes, followed by microtubule disassembly after 5–9 min [20]. Again, it is interesting to note that microtubule depolymerization is a process that has been shown to result in NF-κB activation [21]. Thus, cytoskeletal re-arrangements could represent one of the mechanisms whereby yeast phagocytosis (possibly through CR3) activates NF-κB. In addition to CR3, the ingestion of Y-IgG involves the participation of Fcγ receptors, and recent studies have established a link between Fcγ receptor engagement and NF-κB activation. For instance, cross-linking of Fcγ receptors using agonistic monoclonal antibodies or adherent human IgG was shown to induce NF-κB activation in monocytic THP-1 cells [22]. Similarly, nuclear NF-κB DNA-binding activities were induced in J774 murine macrophage-like cells following culture in the presence of immobilized mouse IgG2a [23]. Thus, our finding that the ingestion of Y-IgG-coated particles results in NF-κB activation might reflect the generation of distinct signals through both Fcγ receptors and CR3.

In conclusion, our data suggest that NF-κB activation could represent one of the molecular bases underlying the inducible expression of κB-dependent genes (and in particular those encoding TNFα and IL-8) observed in peripheral blood neutrophils undergoing the phagocytosis of yeasts or related products, such as zymosan [7–9,24,25]. Consistent with this conclusion is that in phagocytosing neutrophils, activation of NF-κB is an early event which directly precedes the accumulation of TNFα and IL-8 mRNA transcripts. In this regard, previous studies from our laboratory have established that Y-IgG phagocytosis up-regulates IL-8 and TNFα mRNA

steady-state levels within 45–60 min [7,9]. It must be stressed however, that Y-IgG represents a stronger stimulus than LPS for the early accumulation IL-8 and TNFα mRNA transcripts in neutrophils [7–9], while we show herein that phagocytic particles activate NF-κB to a lesser extent than LPS. This therefore indicates that additional processes (aside from NF-κB activation) are involved in the regulation of cytokine and chemokine gene expression in phagocytosing neutrophils. In a broader context, the ability of phagocytic particles to activate NF-κB in neutrophils raises the possibility that other phagocytes might similarly respond to the same stimuli. In particular, monocytes and macrophages are known to ingest a variety of targets, resulting in an enhanced expression of κB-responsive genes such as those encoding TNFα, IL-1β, IL-6, and IL-8 [7–9,24,26]. Moreover, the engagement of receptors involved in phagocytosis leads to NF-κB activation in both monocytic and macrophage-like cell lines [19,22,23]. Studies are in progress to determine whether NF-κB activation is a general consequence of phagocytosis in various cell types.

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