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Activation of the Transcription Factor NF-κB in Lipopolysaccharide-Stimulated U937 Cells

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ABSTRACT. During the course of serious bacterial infections, lipopolysaccharide (LPS) interacts with monocyte/macrophage receptors, resulting in the generation of inflammatory cytokines. Transcription factor NF-κB is crucial in activating the transcription of genes encoding proinflammatory cytokines. In this paper, we demonstrate that the activation of NF-κB by LPS in a promonocytic cell line (U937) followed a rather slow kinetics, depending on the rate of IκB-α inhibitor hydrolysis. No degradation of p105 and p100 inhibitors was observed under these conditions. The transduction pathway leading to NF-κB activation in U937 cells involved the intracellular generation of reactive oxygen species (ROS), as demonstrated by the concomitant inhibitory effects of antioxidants on NF-κB activation and the emission of a fluorescent probe reacting intracellularly with hydrogen peroxide. This ROS pathway was also characterized by the use of other inhibitors. This finding indicates that phospholipase A2 and 5-lipoxygenase are also involved. However, the NF-κB activation pathway involving the acidic sphingomyelinase of the endolysosomial membrane did not seem to participate in the LPS-induced NF-κB activation in U937 cells. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:339–346, 1997.

KEY WORDS. LPS; NF-κB; oxidative stress; signal transduction; monocytes

The major outer membrane component of gram-negative bacteria, LPS,§ triggers a large number of biological responses, including fever, disseminated intravascular coagulation, septic shock and even death [1]. The immune system of the host and in particular the cells of the monocytic lineage play a pivotal role in the mediation of the biological effects of LPS [2–4]. LPS induces these cells to produce cytokines that are detrimental to the host when synthesized in excess [5]. LPS has multiple effects on monocytes/macrophage and is one of the most potent inducers of proinflammatory cytokines such as IL-1β, TNF-α, IL-6 and arachidonic acid metabolites that recruit and activate other immune cells to help fight bacterial infections. Mechanisms by which LPS activates monocyte/macrophage and other cells to induce cytokine expression are now being characterized. The binding of LPS to surface molecules such as CD14 and CD11/CD18 [6, 7] results in increased cytokine mRNA levels through transcriptional and posttranscriptional effects. LPS also increases tyrosine phosphorylation [8] and induces transcription factor NF-κB [9–12]. NF-κB is a rapidly inducible transcription factor that regulates the expression of many genes coding for cytokines, growth factors, acute-phase response proteins and cellular receptors. Thus, NF-κB modulates the cellular response to the applied stimulus (for review, see [13–15]).

NF-κB complexes bind DNA as dimers constituted from a family of proteins designated as the Rel/NF-κB family. In mammals, this family contains the proteins p50, p52, p65 (RelA), RelB and c-Rel [16–20]. NF-κB complexes are sequestered in the cytoplasm of most resting cells by inhibitory proteins belonging to the IκB family [13–17]. Members of the IκB family are IκB-α, IκB-β, p100 and p105 [18–21].

In response to various stimuli, including the interaction of the proinflammatory cytokines TNF-α and IL-1β with their receptors, IκB-α is first phosphorylated on serines 32 and 36 and then rapidly degraded by the proteasome, allowing NF-κB nuclear translocation and gene activation [22–27]. The signal transduction pathways leading to the phosphorylation and degradation of IκB proteins are still poorly understood and even quite controversial. It has been reported that any stimulus activating NF-κB, including TNF-α and IL-1β, proceeds through the intracellular production of ROS [28, 29]. However, a recent report has

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§ *Abbreviations:* BPB, *p*-bromophenacyl-bromide; CHQ, chloroquine; CHX, cyclohemide; DFCH-DA, dichlorofluorescein diacetate; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; IL-1β, interleukin-1β; IL-6, interleukin-6; 5-LOX, 5-lipoxygenase; LPS, lipopolysaccharide; NAC, *N*-acetyl-L-cysteine; NDGA, nordihydroguaiaretic acid; PBS, phosphate buffered saline; PC-PLC, phosphatidylcholine-phospholipase C; PD, polindromic; PDTC, pyrrolidine dithiocarbamate; PKCζ, protein kinase Cζ; PLA2, phospholipase A2; PMA, phorbol-13-myristate acetate; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; TNFR-1, tumor necrosis factor-α type 1 receptor

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indicated that NF-κB could also be activated in the presence of antioxidants or free radical scavengers [30]. Moreover, several studies have indicated that NF-κB activation following TNF-α or IL-1β stimulation can proceed independently of the activation of neutral sphingomyelinase, the production of membrane-associated ceramide and the generation of ROS [10, 31–34].

We studied pathways of NF-κB activation by LPS in a monocytic cell line and report the likely involvement of PLA2, 5-LOX and ROS; activation of the acidic sphingomyelinase is not reported.

MATERIALS AND METHODS

Cell Culture and Reagents

The human promonocytic cell line U937 was obtained from the NIH AIDS Reagent Program (Rockville, MD, USA) and was grown in RPMI 1640 medium (Gibco-BRL, Bethesda, MD, USA) supplemented with 1% antibiotics (streptomycin + penicillin), 1% glutamine and 10% FCS.

LPS (serotype 0111/b4; Sigma, St Louis, MO, USA) stimulation was performed at various concentrations ranging from 0.01 to 1 μg/mL by direct addition to the cell suspension. Before treatment with inhibitors, U937 cells were grown for 1 day in the medium. NAC, PDTC, NDGA, BPB, CHQ (all from Sigma, St Louis, MO, USA), and NH_4Cl (UCB, Brussels, Belgium) were added to the medium either 60 or 120 min before stimulation.

Nuclear and Cytoplasmic Protein Extraction

Nuclear and cytoplasmic protein extracts were prepared as described elsewhere [35]. Cytoplasmic buffer contained 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES pH 7.9), 0.1 mM EDTA, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT and protease inhibitors (Boehringer Protease Inhibitor Kit, Boehringer, Germany). The pelleted nuclei were resuspended in nuclear buffer (50 mM Hepes pH 7.9, 0.05 M KCl, 0.1 mM EDTA, 0.3 M NaCl, 10% glycerol, 1 mM DTT, protease inhibitors), incubated for 5 min at 4°C and centrifuged for 30 min at 14,000 rpm. Protein amounts were quantified with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

Electrophoretic Mobility Shift Assays

EMSAs and supershifting analysis were performed as described elsewhere [35]. The PD-κB probe was

5′-GGTTACAAGGGACTTTCCGCTG-5′-3′
3°-TGTTCCCTGAAAGGCGACGGTT-5′

For supershifting experiments, 1 μL of the antibody was preincubated with the extracts for 30 min on ice before addition of the labeled κB probe.

Immunoblots

Protein extracts were run on a 10% SDS-PAGE gel. After transfer to a nylon membrane (PVDF, Boehringer, Mannheim, Germany) and overnight blocking at 4°C with PBS buffer containing 0.1% (w/v) Tween-20 and 5% dry milk, the membranes were incubated for 1 hr with the first antibody, washed and then incubated with the second peroxidase-conjugated antibody. The reaction was revealed with the enhanced chemoluminescence detection method (ECL kit, Boerhinger, Mannheim, Germany).

Antibodies

The polyclonal IκB-α antibody, the p100/p52 monoclonal antibody, the antipeptide antibody directed against an aminoterminal peptide of p50, and the antipeptide antibody directed against the N-terminal 14 amino acids following the initiator methionine of p65 were kindly provided by Dr. Siebenlist (National Institutes of Health, Bethesda, MD, USA).

Fluorescent Measurement of Intracellular H_2O_2

Formation of H_2O_2 was measured using DFCH-DA [36]. 5×10^6 U937 cells were incubated in 75-cm^2 flasks with 20 μM DCFH-DA in medium 199 (Gibco-BRL, Bethesda, MD, USA) in the dark. After 15 min, the medium was removed, and the cells were washed and incubated with LPS or H_2O_2 for 15 or 30 min. Fluorescence was measured by spectrophotofluorimetry, with excitation at 505 nm and detection of light emission at 525 nm. Cytoplasmic extraction (as described above) was used for hydrogen peroxide determination and the values normalized to protein quantification in nuclear extracts from the same cells.

RESULTS

LPS Influence NF-κB Translocation in U937 Cells with Slow Kinetics

Because NF-κB is inducible by various stimuli in U937 cells [37], we characterized the induction of this transcription factor by LPS in this cell line. EMSA showed that stimulation of U937 cells with LPS at 1 μg/mL induced a strong NF-κB DNA-binding activity, which reached its maximal activation between 60 and 120 min (Fig. 1A) but lasted up to 15 hr. The lowest LPS concentration giving rise to a detectable NF-κB activation was evaluated at 0.1 μg/mL. The kinetics of NF-κB induction by LPS in U937 cells was very different from that initiated by a proinflammatory cytokine such as TNF-α or by phorbol ester. In these latter cases, NF-κB activation could be detected at shorter induc-

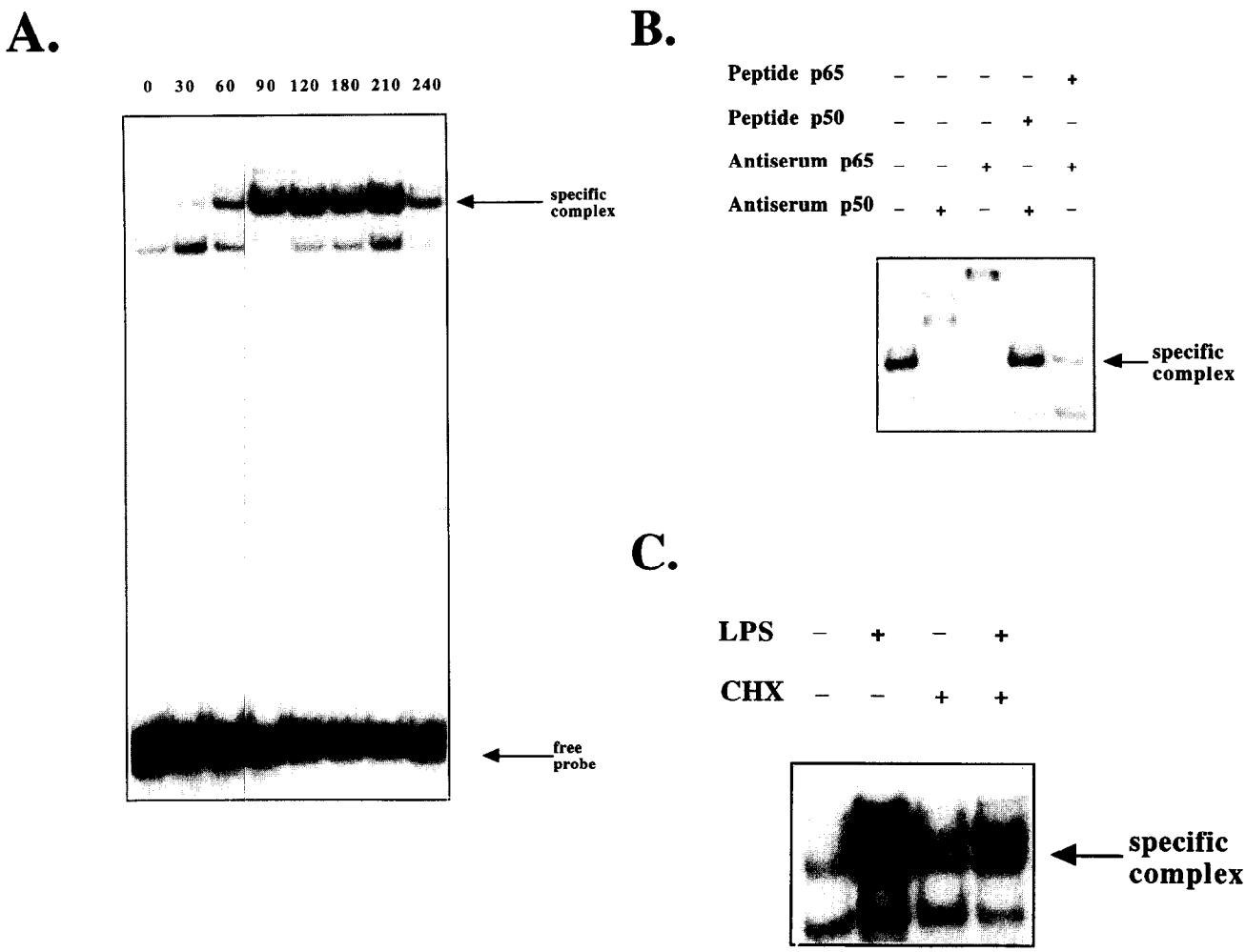

FIG. 1. EMSA analysis of NF-κB activation in LPS-stimulated promonocyte U937 cells. (A) Kinetics of induction of κB-DNA binding activity in LPS-induced cells. Cells were taken at various times after adding LPS (1 μg/mL) and used to prepare nuclear extracts. (B) Immunoreactivity of the LPS-inducible protein-κB DNA complex. Nuclear extracts(5 μg) from U937 cells treated with LPS (1 μg/mL) for 2 hr were either mixed directly with the κB probe or first incubated with antisera specific for p50 or p65 with or without the competitor peptide. (C) Effect of CHX on the induction of κB-DNA binding activity. CHX treatment was carried out by incubating U937 cells with CHX (50 μg/mL) 1 hr before LPS induction.

tion times, being maximal after 30–45 min (data not shown).

Under our experimental conditions, two bands were visible on the autoradiography: an intense band and a fainter, slower-migrating band. Supershifting experiments performed with antibodies directed toward NF-κB proteins demonstrated that the slower band was displaced by both anti-p50 and anti-p65 antibodies and was formed mainly of p50/p65 heterodimers (Fig. 1B). Supershifts induced by these two antibodies could be abolished by the presence of competitor peptides corresponding to the p65 and p50 epitopes, respectively (Fig. 1B).

Because LPS generates the transcriptional activation of several genes encoding proinflammatory cytokines such as IL-1β or TNF-α, we ascertained whether the observed induction of NF-κB was due to the direct action of LPS or was mediated by the extracellular release of cytokines. In the presence of CHX (50 μg/mL), which inhibits *de novo* protein synthesis, NF-κB induction by LPS was unaffected, except for a modest decrease when NF-κB induction was measured after several hours of CHX incubation (Fig. 1C). This experiment therefore ruled out the role of extracellular cytokines in NF-κB induction by LPS.

LPS Induces Processing of IκB-α But Not of p105 and p100

To investigate further the potential pathways for NF-κB activation by LPS, we first confirmed that LPS led to a degradation of the IκB-α protein, as observed on immunoblots performed with cytoplasmic extracts (Fig. 2A). The kinetics of IκB-α degradation paralleled NF-κB activation, with a complete IκB-α degradation observed after 120 min.

LPS treatment of U937 cells did not induce a processing of cytoplasmic p105 IκB-like proteins, as shown on Western blots (Fig. 2B). Likewise, LPS did not induce any detectable processing of p100 molecules (Fig. 2C), demon-

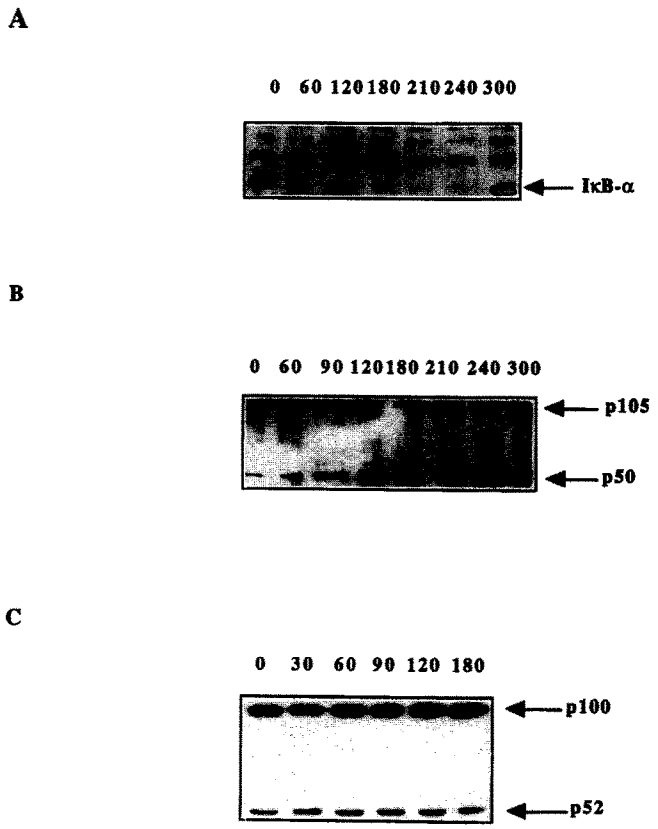

FIG. 2. IκB-α proteolysis (A), p105 (B) and p100 (C) processing in LPS-induced U937 cells. Cells were taken at various times after LPS treatment and used to prepare cytoplasmic extracts. Cytoplasmic proteins (15 μg) were analyzed by SDS-PAGE and transferred on nylon membranes, followed by Western blot analysis using polyclonal IκB-α antibody (A), a p105/p50 monoclonal antibody (B) or an antipeptide p100/p52 polyclonal antibody (C).

strating that in U937 cells LPS-induced NF-κB activation completely originates from IκB-α proteolysis.

Antioxidants Block LPS-Mediated NF-κB Induction in U937 Cells

The kinetics of induction are very similar to those obtained when H_2O_2 is added to either lymphocytes or monocytes [35]. This similarity may suggest that LPS-induced NF-κB activation in U937 cells proceeds through a signal transduction pathway involving ROS. Consistent with previous reports, the free radical scavengers NAC (2.5–30 mM) and PDTC (5–100 μM) completely suppressed NF-κB activation both after LPS stimulation of U937 cells (Fig. 3A,B) and after H_2O_2 treatment of the same cells (data not shown). In both cases, this inhibition was dose dependent and was observed after pretreating the cells for 60 min before induction with LPS for 120 min.

LPS Stimulation of U937 Cells Generates Intracellular H_2O_2

To investigate whether H_2O_2 is produced following LPS stimulation of U937 cells, we used DFCH-DA as a probe

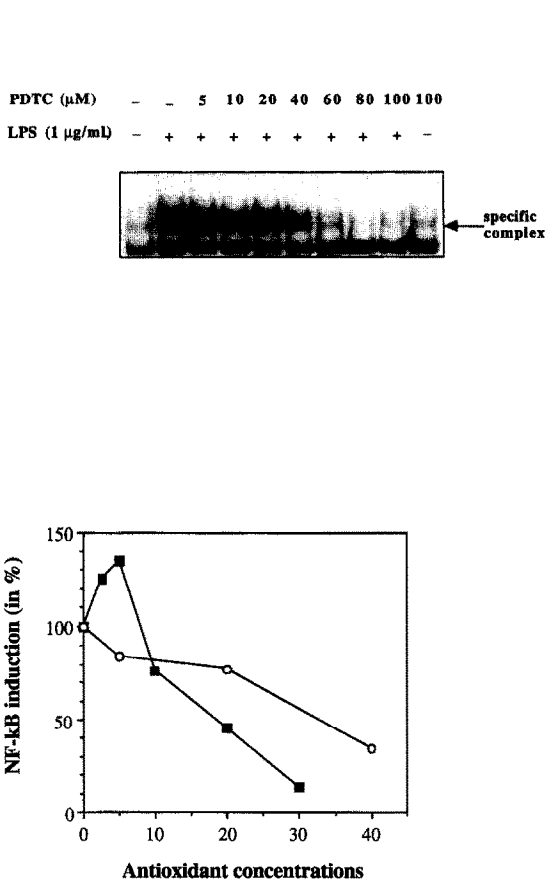

FIG. 3. Effects of antioxidants on NF-κB induction in LPS-induced U937 cells. U937 cells were incubated with various concentrations of PDTC (in μM) (circle) or NAC (in mM) (square) for 1 hr before being induced with LPS (1 μg/mL). Nuclear proteins (5 μg) were prepared 2 hr after induction, analyzed by EMSA (A) and quantified by phosphorimaging (B).

and measured the induced fluorescence [36]. In this experimental setting, we observed a strong H_2O_2 production in U937 cells as early as 2 min after LPS stimulation (Fig. 4), similar to the effect obtained by adding 1 mM H_2O_2 directly to these cells (data not shown). In addition, the LPS-induced H_2O_2 generation was almost completely abolished when the stimulation was carried out in the presence of PDTC at nontoxic concentrations (5–10 μM). These results confirm that LPS-induced NF-κB translocation is mediated in U937 cells by the intracellular generation of H_2O_2.

NF-κB Activation by LPS Proceeds Through the PLA2–5-LOX Pathway

Because the generation of intracellular H_2O_2 could proceed through the involvement of PLA2 and 5-LOX [38], we used inhibitors to test the functional importance of these enzymes in LPS-mediated activation of NF-κB. The involvement of PLA2 was evaluated by using BPB, an irreversible inhibitor of PLA [39]. To this end, U937 cells were pre-

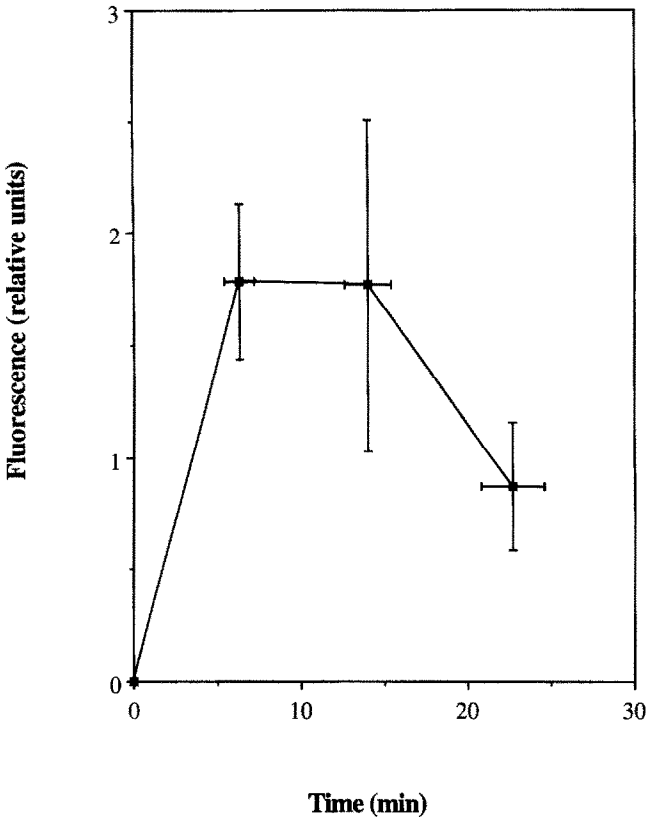

FIG. 4. Intracellular H_2O_2 production after LPS stimulation of U937 cells. Cells were incubated with the fluorescent probe DFCH-DA (20 μM) for 10 min. After washing, the cells were induced with LPS (1 μg/mL) for various times (0–30 min). Fluorescence was measured in cytoplasmic extracts by excitation at 505 nm and emission at 525 nm. Values represent mean ± SD (n = 3).

treated for 60 min with increasing concentrations of BPB and then stimulated with LPS. Nontoxic concentrations of BPB (1–10 μM) strongly inhibited NF-κB activation by LPS in a dose-dependent manner, demonstrating the importance of PLA2 in the pathway (Fig. 5A). The involvement of 5-LOX was evaluated by the use of NDGA at nontoxic concentrations (3–50 μM). U937 cells were pretreated for 60 min with NDGA and then stimulated with LPS for 120 min. NF-κB activation was also diminished in a dose-dependent manner by NDGA (Fig. 5B), demonstrating the importance of the PLA2–5-LOX pathway in the activation of NF-κB.

An alternative pathway could proceed through the acidic sphingomyelinase. This pathway requires the endocytosis of surface receptors, the activation of PC-PLC, the acidic sphingomyelinase and probably several protein kinases, including PKCζ [4, 15, 32, 34, 40]. Two inhibitors of acidic sphingomyelinase, NH_4Cl (6–60 mM) and CHQ (5–100 μM), were tested. These two reagents specifically block the acidic sphingomyelinase pathway without affecting neutral sphingomyelinase and the PLA2–5-LOX pathway [15, 41]. Neither NH_4Cl nor CHQ were able to block NF-κB induction efficiently by LPS (Figs. 5C,D).

DISCUSSION

The identification of the signal transduction pathways leading to NF-κB activation after the interaction of proinflammatory mediators with their receptors has been the subject of many investigations. There is some evidence in the literature that more than one pathway can account for TNF-α- or IL-1β-mediated NF-κB activation. Several reports have claimed that NF-κB activation depends on an activation of 5-LOX and on oxidative stress [28, 29, 38], and others have demonstrated that the acidic sphingomyelinase pathway is necessary and sufficient for NF-κB induction after TNF-α stimulation [15]. Similarly, different investigators have reported that ceramide is a major second messenger for TNF-α-induced NF-κB activation [41–46], and others have denied any role for ceramide in IκB-α degradation [31, 33, 46]. In this study, we report that LPS induces NF-κB in a monocytic cell line *via* a pathway involving the generation of ROS.

This work shows that in the U937 cell line LPS stimulates the intracellular production of ROS and that NF-κB activation depends on this oxidative stress. This finding is consistent with previous reports demonstrating that NF-κB activation by IL-1β or TNF-α can be inhibited by antioxidants and that products of 5-LOX can directly active NF-κB [28, 29, 38, 47]. in these cells, we can postulate that a major pathway for NF-κB induction after LPS stimulation acts through the activation of neutral sphingomyelinase, the production of ceramide in the plasma membrane and the activation of PLA2 and 5-LOX with the activation of the latter enzyme subsequently leading to the production of ROS. This pathway (or a part of it) also seems to be active in mesangial cells, where ROS are involved in cyclooxygenase-2 expression after activation by IL-1β, TNF-α or LPS [48].

These ROS are the cofactors for TNF-α-dependent induction of c-fos, activation of manganese superoxide dismutase, cytotoxicity and, at least in some cell types, NF-κB activation, but their role following LPS interaction with its receptor is still unclear. A previous report has stated that LPS treatment of HeLa cells results in activation of NF-κB with slow kinetics [37, 49] but also through the participation of IκB [21].

TNF-α and IL-1β may stimulate similar signal transduction pathways, and most studies have investigated signaling after TNF-α interaction with its 55-kDa TNFR-1. However, TNF-α and IL-1β exert different biological functions, and their signaling pathways are thus probably partly distinct. The activation of NF-κB depends on different pathways. Stimulation of epithelial cells such as OVCAR-3 cells by H_2O_2 leads to a slow activation of NF-κB following partial degradation of IκB-α, p100 and p105 [50]. Stimulation of the same cells by IL-1β activates NF-κB at a much faster rate and proceeds exclusively through a complete degradation of IκB-α, which does not involve intracellular oxidative stress [50]. These differences in the kinetics and pathways of NF-κB activation might translate into specific

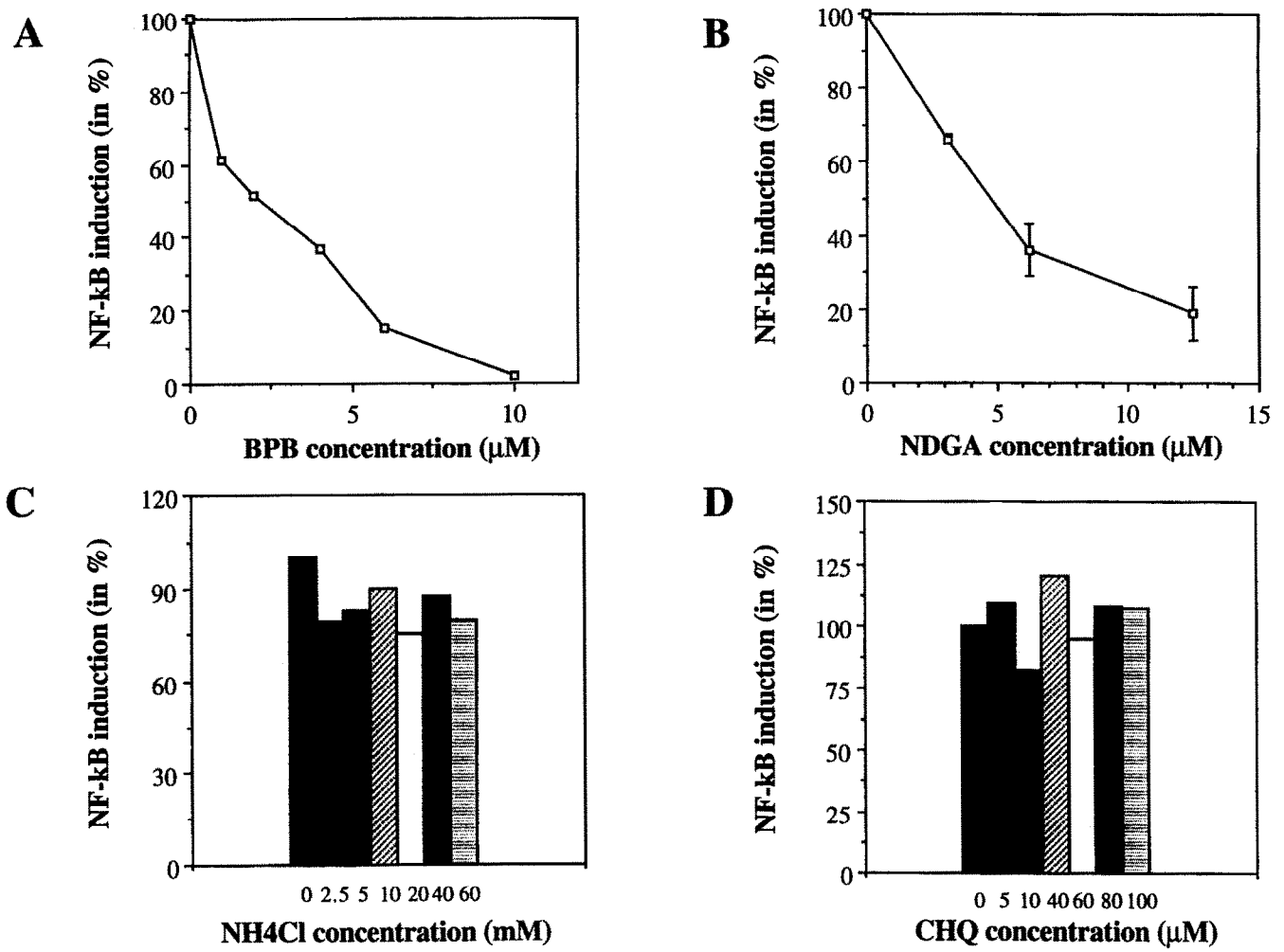

FIG. 5. Effects of various inhibitors on LPS-induced NF-κB activation in U937 cells. One hour before LPS stimulation (1 μg/mL), U937 cells were incubated with various concentration of BPB (A), NDGA (B), NH_4Cl (C) and CHQ (D). Nuclear extracts were prepared 2 hr after LPS induction and analyzed by EMSA. The κB-DNA binding activities were quantified by phosphorimaging.

functions, allowing NF-κB to be activated simultaneously with other transcription factors, resulting in the transcription of distinct genes.

The nature of the induced NF-κB complexes might also differ following different stimuli. In this study, we used only anti-p50 and anti-p65 antibodies to examine the specificity of the induced NF-κB complexes observed on EMSA. Other NF-κB-like complexes may be induced simultaneously or with specific kinetics. Every NF-κB complex harbors specific affinities for distinct κB sites and might therefore preferentially influence distinct promoters [51, 52]. The observation that U937 cells respond to LPS by the generation of intracellular ROS and the nuclear translocation of NF-κB is strong evidence that signaling molecules are similar to those present in lymphocytes induced by TNF-α or PMA. Characterization of the transduction pathway triggered by LPS is of the utmost importance because LPS is a very potent activator of the host immune response including induction of inflammatory mediator [1]. Although activation of PLA2 and 5-LOX by LPS should be confirmed as an intermediate step in the pathway leading to NF-κB translocation, these enzymes catalyse the release of potent proinflammatory mediators [53, 54]. Designing drugs that are potent inhibitors of PLA2 [55] or 5-LOX [56] will constitute an important step in characterizing the intermediates activated by LPS and contribute to a better understanding of the precise involvement of these enzymes in inflammatory processes [57].

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