

Wortmannin inhibits activation of nuclear transcription factors NF- κ B and activated protein-1 induced by lipopolysaccharide and phorbol ester

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Abstract Whether all inflammatory agents activate nuclear transcription factors NF- κ B and activated protein-1 (AP-1) through the same mechanism is not known. We examined the effect of the phosphatidylinositol-3-kinase (PI-3K) inhibitor wortmannin on the activation of NF- κ B and AP-1 by different inflammatory agents. Wortmannin blocked NF- κ B and AP-1 activation by lipopolysaccharide and phorbol ester but had minimal effect on activation by hydrogen peroxide, ceramide, okadaic acid and tumor necrosis factor. Inhibition of NF- κ B correlated with abrogation of the degradation of I κ B α and of NF- κ B-dependent reporter gene transcription. Thus, the mechanism of NF- κ B and AP-1 activation by lipopolysaccharide and phorbol ester involves PI-3K.

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Key words: Lipopolysaccharide; Phorbol ester; Nuclear transcription factor- κ B; Activated protein-1; Wortmannin; Phosphatidylinositol-3-kinase

1. Introduction

Inflammation is an essential host-defense mechanism that is induced in response to a wide variety of exogenous and endogenous stimuli. These include radiation, lipopolysaccharide (LPS), products of gram-negative bacteria, cytokines (such as tumor necrosis factor (TNF) and interleukin-1 (IL-1)), ceramide, H₂O₂, phorbol ester (PMA), hyperoxia/hypoxia, chemotherapeutic agents and okadaic acid (OA). When cells are exposed to inflammatory agents, a number of genes are activated, including those encoding cell surface adhesion proteins, cyclooxygenases, lipooxygenases, inflammatory cytokines and their receptors, components of the major histocompatibility complex and metalloproteases. The promoter region of most of these genes contains binding sites for nuclear transcription factor- κ B (NF- κ B). Activation of this factor leads to the expression of these inflammatory genes. Most of the inflammatory stimuli listed in fact activate NF- κ B (for references see [1]).

Under normal conditions, NF- κ B is found in the cytoplasm

of all eukaryotic cells as a heterotrimer consisting of p50, p65 and an inhibitory subunit of NF- κ B (I κ B α). When cells are exposed to inflammatory agents, within minutes in most cases I κ B α undergoes phosphorylation, ubiquitination and degradation, thus exposing the nuclear localization signals on p50 and p65, which in turn results in translocation of the p50–p65 heterodimer to the nucleus. This activated heterodimer then binds to a unique decameric nucleotide sequence present in the promoter of the inflammatory genes, leading to gene expression.

Although the phosphorylation of I κ B α at serines 32 and 36 is considered a critical step, the pathway that leads to it is incompletely understood. Three different kinases, referred to as NF- κ B-inducing kinase, IKK- α , and IKK- β , that are involved in the phosphorylation of I κ B α have recently been identified. However, it is not clear how these kinases are interrelated and how they phosphorylate I κ B α (for references see [2]). These kinases are activated in response to TNF, but whether they are activated in response to other inflammatory stimuli is also not clear nor is the kinase activation pathway. Protein tyrosine kinases, protein tyrosine phosphatases, reactive oxygen intermediates and ceramide have also been implicated in this pathway [3–6].

Most agents that activate NF- κ B also activate activated protein-1 (AP-1), another transcription factor [7]. Like NF- κ B, AP-1 is also a heterodimer, consisting of c-Fos and c-Jun proteins. The activation of AP-1 by inflammatory stimuli involves induction of *c-fos* synthesis and the phosphorylation of both c-Fos and c-Jun [8].

By using wortmannin [9], which blocks the enzymatic activity of phosphatidylinositol-3-kinases (PI-3K), it has been shown that PI-3K plays a role in AP-1 activation by epidermal growth factor, IL-1, and PMA [10–12]. CD28-mediated AP-1 activation in T cells also requires PI-3K [13]. The role of PI-3K in the activation of NF- κ B is less clear. Recent reports indicate that wortmannin has no effect on CD28-mediated NF- κ B activation but that activated by IL-1 is blocked [12,13], thus suggesting there is a difference in the pathways. Whether wortmannin affects NF- κ B and AP-1 activation induced by other inflammatory stimuli is not known.

In the present report, we investigated the effects of wortmannin on the activation of AP-1 and NF- κ B by TNF, LPS, PMA, ceramide, H₂O₂ and OA. Our results indicate that wortmannin blocks NF- κ B and AP-1 activation induced by LPS and PMA but not that activated by TNF, ceramide, H₂O₂ or OA. NF- κ B-dependent reporter gene expression was also differentially regulated thus suggesting that NF- κ B and AP-1 are activated by different pathways when exposed to different agents.

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Abbreviations: LPS, lipopolysaccharide; PMA, phorbol ester; OA, okadaic acid; TNF, tumor necrosis factor; AP-1, activated protein-1; IL-1, interleukin-1; SA-LPS, serum-activated LPS; NF- κ B, nuclear transcription factor- κ B; I κ B, inhibitory subunit of NF- κ B; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; PI-3K, phosphatidylinositol-3-kinase

2. Materials and methods

2.1. Materials

Wortmannin, glycine, H7, PMA, LPS (*Escherichia coli*, 055:B5), H₂O₂, OA, ceramide (C-2) and chloramphenicol were obtained from Sigma (St. Louis, MO, USA). All other reagents were obtained as previously described [6].

2.2. Cell lines

The cell lines U937 (human histiocytic lymphoma), Jurkat (human T-cell), HeLa (human epithelial) and H4 (human glioma) were obtained and cultured as described [6].

2.3. Assay for NF- κ B and AP-1

Activated NF- κ B and AP-1 were assayed following the method as described [6,15]. A 45-mer double-stranded NF- κ B oligonucleotide from the HIV-LTR, 5'-TTGTTACAAGGGACTTCCGCTG GGGACTTTC CAGGGA GGCCT GG-3' (bold indicates NF- κ B binding sites) was used for NF- κ B binding. To examine the specificity of binding of NF- κ B to the DNA, a double-stranded mutated oligonucleotide, 5'-TTGTTACAA CTCACTTTC CGTGCTCACTTTC-CAGGGAGG CGTGG-3', was used.

To assay for AP-1 activation, the ³²P-end-labeled AP-1 consensus oligonucleotide 5'-CGCTTGA TGACTCA GCCGGAA-3' (bold indicates AP-1 binding site) was used.

2.4. Western blot

After the NF- κ B activation reaction described above, postnuclear extracts were resolved on 8.5% Sodium dodecyl sulfate–polyacrylamide gels, electrotransferred and probed with a rabbit polyclonal antibody against I κ B α and p65 and detected by chemiluminescence (ECL, Amersham) [16].

2.5. Transient transfection and chloramphenicol acetyltransferase (CAT) assay

NF- κ B-dependent gene expression was measured by a transient transfection of U937 cells with -243RMICAT (wild) and -243RMICAT-km (mutant) genes [14] for 6 h by the calcium phosphate method at 37°C and then treated with wortmannin (10 nM) for 1 h before stimulation with 0.1 and 1 μ g/ml of serum-activated (SA)-LPS or 10 and 25 ng/ml of PMA for 1 h. Thereafter, the cells were washed with phosphate-buffered saline and examined for CAT activity as described [17].

3. Results

Whether PI-3K plays any role in the pathway leading to activation of NF- κ B and AP-1 was examined using wortmannin and a wide variety of NF- κ B and AP-1 inducers. The time of incubation and the concentration of wortmannin used in our studies had no effect on cell viability as determined by the trypan blue dye uptake method (data not shown).

3.1. Wortmannin blocks NF- κ B activated selectively by PMA and SA-LPS

The results shown in Fig. 1A indicate that wortmannin completely blocked the activation of NF- κ B induced by SA-LPS and PMA, had a partial effect on activation by ceramide and no effect on activation by H₂O₂, OA and TNF. Thus there must be a difference in the pathways leading to NF- κ B activation induced by different agents.

To show that the retarded band visualized by electrophoretic mobility shift assay (EMSA) in SA-LPS-treated cells was indeed NF- κ B, we incubated nuclear extracts from SA-LPS-activated cells with antibody (Ab) to either p50 (NF- κ BI) or p65 (Rel A) subunits and then conducted EMSA. Antibodies to either subunit of NF- κ B retarded the mobility and shifted the band to a higher molecular weight (Fig. 1B), thus suggesting that the SA-LPS-activated complex consisted of p50 and

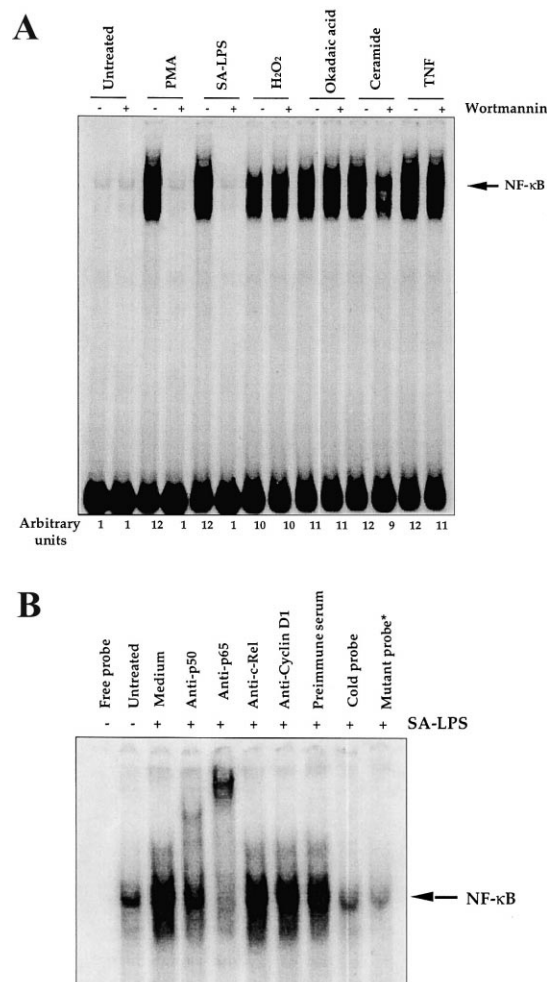


Fig. 1. A: Effect of wortmannin on different activators (PMA, SA-LPS, H₂O₂, OA, ceramide and TNF) of NF- κ B. U937 cells (2×10^6 /ml) were preincubated for 1 h at 37°C with wortmannin (5 nM) followed by PMA (25 ng/ml), SA-LPS (100 ng/ml), H₂O₂ (250 μ M), OA (500 nM), ceramide (10 μ M) or TNF (0.1 nM) for 30 min and then tested for NF- κ B activation as described in Section 2. B: NF- κ B is composed of p50 and p65 subunits. Nuclear extracts from TNF-treated cells were incubated with anti-p50, anti-p65, anti-c-Rel, anti-cyclin D1, ceramide (10 μ M) or unlabeled probe for 1 h at room temperature and then analyzed for NF- κ B by EMSA as described. To determine the specificity, the oligo with mutated NF- κ B binding site was labeled and used for binding.

p65 subunits. Neither preimmune serum nor the irrelevant antibodies anti-c-Rel and anti-cyclin D1 had any effect on the mobility of NF- κ B. In addition the results show that the NF- κ B band disappeared in the presence of excess (100-fold) cold NF- κ B probe, and an oligonucleotide with mutated NF- κ B site failed to bind to the NF- κ B protein, thus all together indicating the composition and specificity of binding of NF- κ B to the DNA.

3.2. Dose-response of inhibition by wortmannin of NF- κ B activation

To determine the minimum amount of wortmannin required to block LPS- and PMA-induced NF- κ B activation, U937 cells were preincubated for 1 h with different concentrations of wortmannin followed by treatment with SA-LPS (100 ng/ml) or PMA (25 ng/ml) for 30 min at 37°C and then examined for NF- κ B activation by EMSA. The results in Fig.

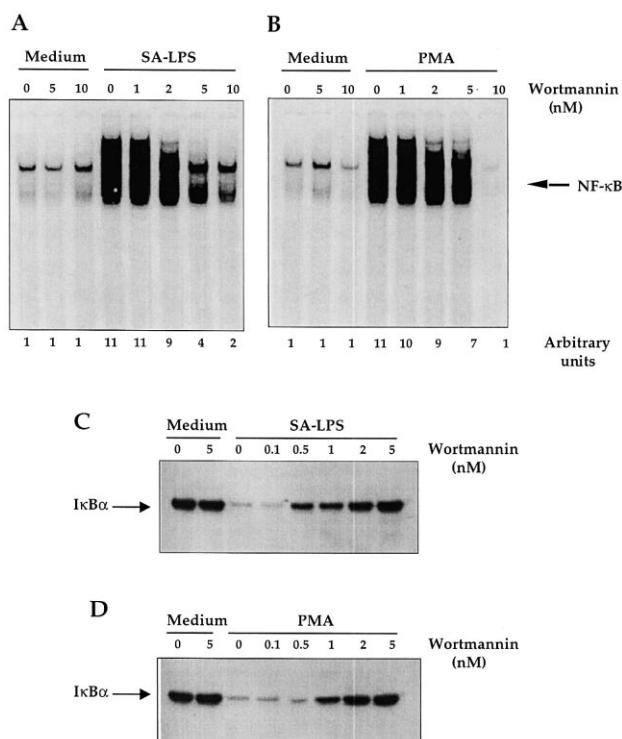


Fig. 2. Effect of different concentrations of wortmannin on induction of NF-κB by SA-LPS (A) and PMA (B). U937 cells (2×10^6 /ml), pretreated with different concentrations of wortmannin for 1 h at 37°C, were incubated with SA-LPS (A) or PMA (B) for 30 min and then assayed for NF-κB by EMSA. C and D: Effect of wortmannin on degradation of IκBα. U937 cells (2×10^6 /ml), pretreated with different concentrations of wortmannin for 1 h at 37°C and incubated with SA-LPS (C) or PMA (D) for 15 min and assayed for IκBα in cytosolic fractions by Western blot analysis.

2A indicate that 10 nM wortmannin inhibited almost 90% of the SA-LPS-induced NF-κB activation. The same concentration of wortmannin had a more dramatic effect on PMA-induced NF-κB activation (Fig. 2B) reducing NF-κB activation below basal levels.

3.3. Wortmannin inhibits SA-LPS- and PMA-dependent degradation of IκBα

To determine whether the inhibitory action of wortmannin was due to its effect on IκBα degradation, the cells were pretreated with different concentrations of wortmannin for 1 h and then stimulated with either SA-LPS or PMA for 15 min, the cytoplasmic extracts were prepared and examined for levels of IκBα by Western blot analysis. The IκBα was degraded completely within 15 min by SA-LPS or PMA in untreated U937 cells (data not shown). With increased concentrations of wortmannin there was a decrease in the degradation of IκBα induced by SA-LPS (Fig. 2C) or by PMA (Fig. 2D). A 50% decrease with 0.5 nM and 100% protection at 5 nM of wort-

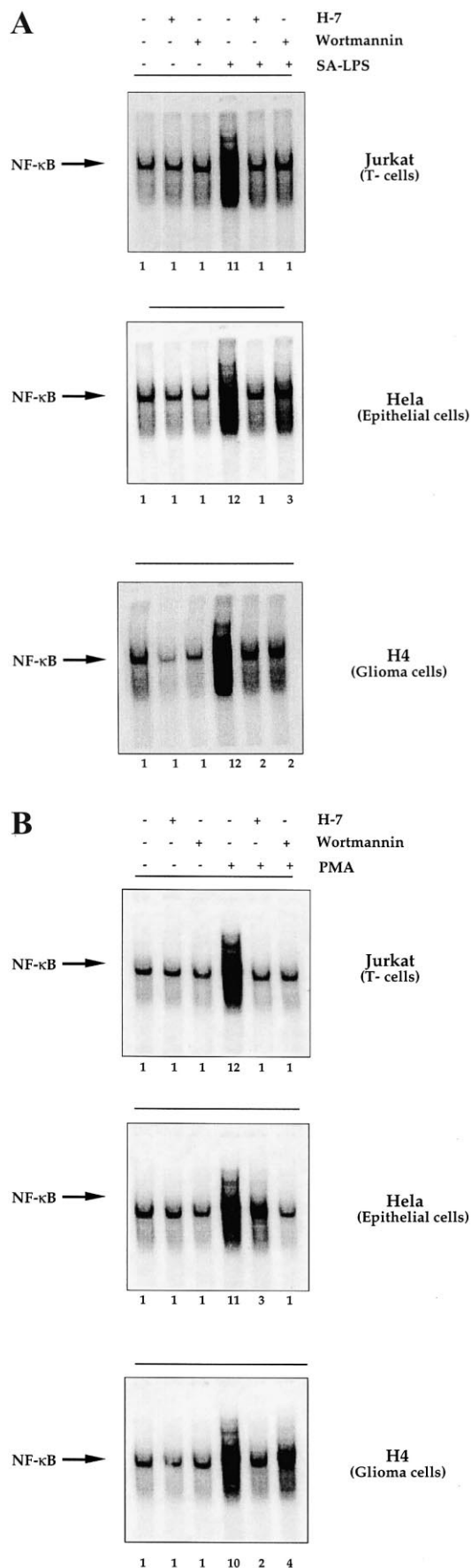


Fig. 3. Effect of wortmannin and H7 on activation of NF-κB induced by SA-LPS (panel A) and PMA (panel B) in different cell lines. Jurkat, HeLa and H4 cells (2×10^6 /ml) were pretreated with wortmannin (5 nM) and H7 (50 nM) for 1 h at 37°C and then incubated with 100 ng/ml SA-LPS (panel A) or 25 ng/ml PMA (panel B) for 30 min. After these treatments nuclear extracts were prepared and then assayed for NF-κB.

mannin was observed in the case of SA-LPS stimulation (Fig. 2C). In the case of PMA, 1 nM wortmannin gave about 50% protection and complete protection was observed at 5 nM (Fig. 2D). Wortmannin by itself had no effect on the constitutive levels of I κ B α . These results indicate that wortmannin blocked NF- κ B activation by blocking I κ B α degradation.

3.4. Inhibition of NF- κ B activation by wortmannin is not cell type-specific

All the effects of wortmannin described so far were observed in U937 cells. Since the NF- κ B activation mechanism has been shown to differ between epithelial and lymphoid cells [18], we investigated whether wortmannin affects NF- κ B activation in T-cells (Jurkat) and epithelial (HeLa) and glioma (H4) cells. Wortmannin inhibited NF- κ B activation in all cell types by both SA-LPS (Fig. 3A) and PMA (Fig. 3B). In all cell types H7, a protein kinase C (PKC) inhibitor, blocked both LPS- (Fig. 3A) and PMA-induced (Fig. 3B) NF- κ B activation. These results suggest that like PMA, LPS may also activate NF- κ B through induction of PKC.

3.5. Wortmannin represses NF- κ B-CAT reporter gene expression

We investigated whether wortmannin blocked NF- κ B-dependent gene expression. The promoter of the MDR gene containing a NF- κ B site linked to a CAT reporter gene was

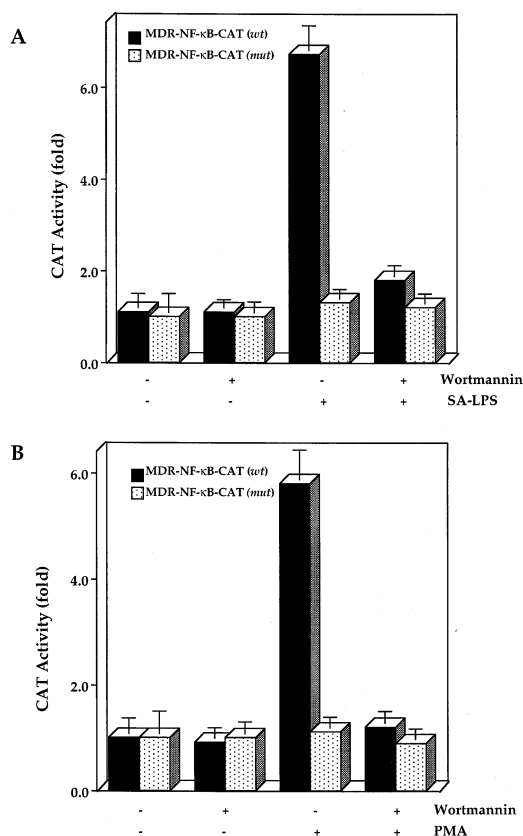


Fig. 4. Effect of wortmannin on the NF- κ B-dependent CAT reporter gene expression induced by SA-LPS and PMA. Cells were transiently transfected with MDR-NF- κ B-CAT (-243RMICAT), both wild (wt) and mutant (mut), treated with 5 nM wortmannin for 1 h, exposed to 100 ng/ml SA-LPS (A) and 25 ng/ml PMA (B) for 1 h and then assayed for CAT activity as described in Section 2. Results are expressed as fold activity over the non-transfected control.

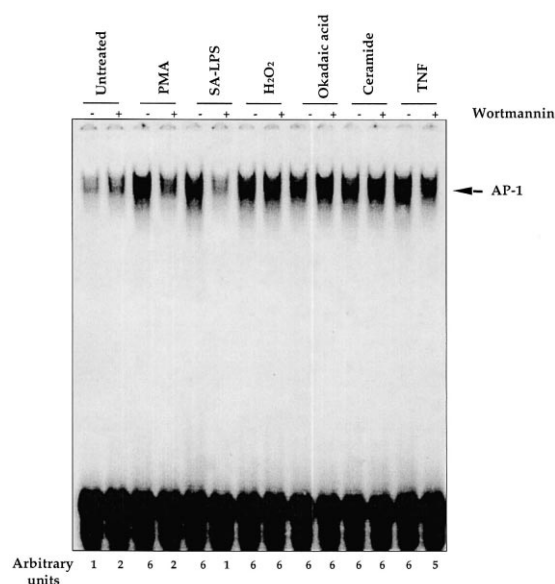


Fig. 5. Effect of wortmannin on different activators (PMA, SA-LPS, H₂O₂, OA, ceramide and TNF) of AP-1. U937 cells (2×10^6 /ml) were pre-incubated for 1 h at 37°C with wortmannin (5 nM) followed by PMA (25 ng/ml), SA-LPS (100 ng/ml), H₂O₂ (250 μ M), OA (500 nM), ceramide (10 μ M) or TNF (0.1 nM) for 30 min and then tested for AP-1 activation as described in Section 2.

used to transiently transfect the cells, the cells were pretreated with wortmannin and then they were stimulated with SA-LPS or PMA and assayed for CAT expression. As expected, an almost seven-fold increase in CAT activity occurred upon stimulation with 100 ng/ml concentration of SA-LPS (Fig. 4A). This activity was reduced by greater than 90% on pretreatment of cells with wortmannin for 1 h. About a six-fold increase in CAT expression was observed in PMA-treated cells and this increase was completely abolished by wortmannin (Fig. 4B). The transfection of cells with plasmids that had a mutated NF- κ B-binding site did not result in induction of CAT activity by either SA-LPS or PMA, thus indicating that this effect was dependent on NF- κ B. These results demonstrate that wortmannin also blocked NF- κ B-dependent gene expression.

3.6. Wortmannin inhibits SA-LPS- and PMA-induced AP-1 activation

Most of the agents that activate NF- κ B also activate the transcription factor AP-1 [7]. Therefore, we examined the effect of wortmannin on activation of AP-1 by PMA, SA-LPS, H₂O₂, OA, ceramide and TNF. As shown in Fig. 5, all five agents activated AP-1. However, only SA-LPS- and PMA-induced AP-1 activation was blocked by wortmannin.

In U-937 cells, SA-LPS activated AP-1 in a dose-dependent manner reaching its maximum of about seven-fold at a concentration of 100 ng/ml SA-LPS (Fig. 6A). PMA activated AP-1 by seven-fold at 25 ng/ml dose (Fig. 6B). The minimum concentration of wortmannin required to inhibit LPS- and PMA-induced activation of AP-1 was found to be 5 nM, a dose that suppressed activation induced by both SA-LPS (Fig. 6C) and PMA (Fig. 6D) by 90%. These results indicate that wortmannin blocks not only NF- κ B activation but also AP-1 activation induced by LPS and PMA.

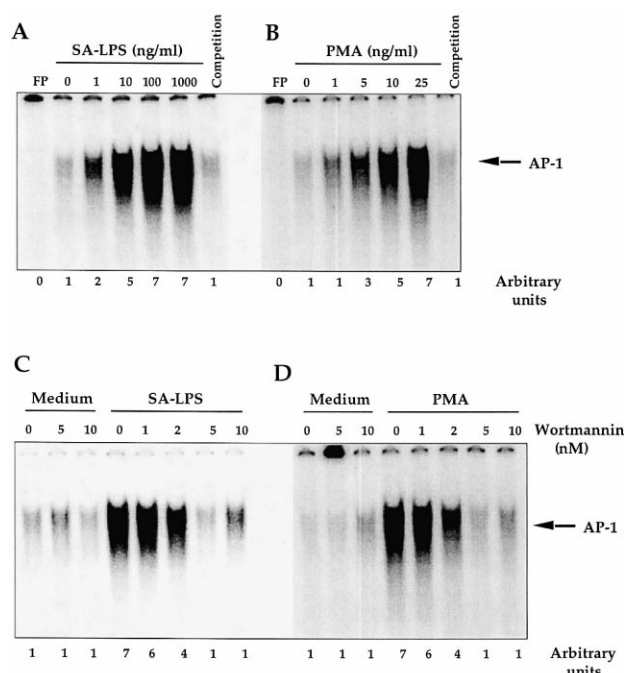


Fig. 6. Effect of wortmannin on SA-LPS- and PMA-dependent AP-1 activation. U937 cells (2×10^6) were incubated with different concentrations of SA-LPS (A) and PMA (B) and then AP-1 expression was assayed by EMSA. Cells were pre-incubated with different concentrations of wortmannin and then stimulated with 100 ng/ml SA-LPS (panel C) or 25 ng/ml PMA (panel D) for 1 h. Then AP-1 was assayed as described in Section 2.

4. Discussion

Our results show that wortmannin blocked NF- κ B activation induced by LPS and by PMA, but had no significant effect on H_2O_2 -, OA- or TNF-induced NF- κ B activation. Ceramide-induced NF- κ B activation was partially affected. The I κ B α degradation needed for nuclear translocation of NF- κ B was completely blocked by the PI-3K inhibitor. This correlated with NF- κ B-dependent reporter gene expression. The inhibitory effect of wortmannin was not cell type-specific.

The various steps that lead to NF- κ B activation are not fully known. Evidence is emerging, however, which indicates that different agents may activate NF- κ B through divergent pathways. For instance I κ B β has profound effects on the activation of NF- κ B by LPS but not that by PMA or TNF [19]. Our results support the notion that different agents activate NF- κ B through different mechanisms. For instance, PI-3K may be needed for NF- κ B activation by LPS and PMA but not for activation by other agents. That PI-3K is required for NF- κ B activation by LPS and PMA was also confirmed from our independent studies in which we used cells from p85 (one of the subunits of PI-3K) gene-knock out mice (Manna and Aggarwal, manuscript in preparation). Recently, it was shown that PI-3K is also needed for activation of NF- κ B by IL-1 [12]. Wortmannin, however, like TNF, H_2O_2 and OA, had no effect on CD28-mediated NF- κ B activation in T cells [13]. Whether wortmannin inhibits IL-1-induced NF- κ B activation through abrogation of I κ B α degradation was not reported [13]. Our results show that wortmannin inhibited NF- κ B activation by blocking the degradation of I κ B α . How wortmannin blocks I κ B α degradation is not clear. It may block phos-

phorylation and ubiquitination, an essential step that precedes degradation [1], or another step upstream to it. Recently, three different kinases have been implicated in I κ B α phosphorylation, but whether those kinases are also needed for NF- κ B activation by LPS and PMA is not known [2]. We found that wortmannin also blocked NF- κ B-dependent reported gene expression induced by PMA and LPS. This is consistent with previous results on IL-1 [12]. Recently, the role of PI-3K in NF- κ B activation by bradykinin, pervanadate and IL-1 was also reported [12,20–22]. TNF has been found to activate PI-3K [23]. Recently several reports have appeared which show that NF- κ B activation requires a PI-3K-Akt pathway [24–26]. Our results with LPS- and PMA-induced NF- κ B activation are consistent with these reports. Our results also indicate that the activation of NF- κ B by both LPS and PMA is sensitive to H7, a specific inhibitor of protein kinase C (PKC). That PKC could play a synergistic role with PI-3K has been demonstrated [27]. Whether PKC directly activates PI-3K, however, is not well understood.

We found that besides NF- κ B, PI-3K may also play an important role in AP-1 activation by LPS and PMA. The role of PI-3K in PMA-induced AP-1 activation is in agreement with a recent report [11]. LPS is known to activate PI-3K [28] and our results show that it is needed for AP-1 activation by LPS. Like NF- κ B, PI-3K is needed for IL-1-induced AP-1 activation [12]. In addition, EGF-induced AP-1 activation requires PI-3K [10]. The identity of the various intermediates involved in AP-1 activation are not fully known. Both phospholipase D (PLD) and protein kinase C have roles in CD3-mediated AP-1 activation in T cells [21]. Since PLD-induced AP-1 activation is also blocked by wortmannin, it may be that PI-3K is situated downstream of PLD [29]. Overall our results demonstrate that PI-3K plays a critical role in the activation of both NF- κ B and AP-1 by LPS and PMA but not by other agents. These studies provide an additional target to design anti-inflammatory agents for diseases mediated through NF- κ B activation.

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