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Herbimycin A blocks IL-1-induced NF-κB DNA-binding activity in lymphoid cell lines

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The mechanism by which interleukin-1α (IL-1α) activates NF-κB DNA-binding activity is not completely understood. While it is well established that protein kinase C can activate NF-κB, neither protein kinase C nor protein kinase A appears to be critical in the induction of NF-κB by IL-1α. Since a number of growth factors signal via protein tyrosine kinase, in this study we examined a possible involvement of protein tyrosine kinase in the IL-1α-induced NF-κB. The results showed that in the murine pre-B cell line 70Z/3 and in the murine T cell line EL-4 6.1 C10 IL-1α-induced NF-κB was associated with transient increase in protein tyrosine kinase activity. Pre-treatment of these cell lines with herbimycin A, an inhibitor of tyrosine kinase activity, blocked the IL-1α-enhanced protein tyrosine kinase activity and the IL-1α-induced NF-κB DNA-binding activity. Herbimycin A at concentrations sufficient to block IL-1α-induced NF-κB did not block the phorbol 12-myristate 13-acetate (PMA)-induced NF-κB. The data suggest that IL-1α and PMA activate NF-κB by different pathways and that induction of NF-κB DNA-binding activity by IL-1 might be dependent on protein tyrosine phosphorylation.

Herbimycin A; IL-1α; NF-κB; B lymphocyte; T lymphocyte

1. INTRODUCTION

The pre-B lymphocyte cell line 70Z/3 and the T-lymphoma cell line EL-4 6.1 C10 have proven to be useful systems to study the biological effects of IL-1. In 70Z/3, IL-1α induces expression of the κ immunoglobulin light chain gene while in EL-4 6.1 C10 IL-1α stimulates expression of IL-2 [1–3]. Expression of the κ light chain and IL-2 genes can be regulated by the ubiquitous transcription factor NF-κB [2–4]. In most resting cells, NF-κB is located in the cytoplasm in an inactive state complexed with an inhibitor, IκB. Treatment of cells with phorbol 12-myristate 13-acetate (PMA) causes the dissociation of this complex, allowing NF-κB to translocate to the nucleus where it binds to its cognate DNA element [5,6]. Based on this observation it was postulated that protein kinase C can activate NF-κB DNA-binding activity in vivo. This postulate was further supported by the observation that phosphorylation in vitro of IκB by protein kinase C blocks its ability to inhibit NF-κB DNA-binding activity [7]. Although there is strong evidence that phorbol ester activates NF-κB via a protein kinase C-dependent pathway, the mechanisms mediating activation of NF-κB by physiological inducers have not been defined [1,7–9]. For example, while IL-1 and tumor necrosis factor (TNF) activate protein kinase C [8–10], the induction of NF-κB by these physiological agents is independent of protein kinase C [8,9]. The role of cAMP in the lymphokine-induced NF-κB has also been examined. Shirakawa et al. reported that IL-1 increases intracellular levels of cAMP and concluded that protein kinase A is a key step in the pathway leading to activation of NF-κB [11]. However, in contrast to this study, two laboratories have now shown that IL-1α-stimulated cAMP is not sufficient to fully activate NF-κB [1,12]. Thus, mechanisms other than those involving protein kinase A or protein kinase C must trigger IL-1-induced activation of NF-κB.

A number of important physiological agents signal through protein tyrosine kinases [13]. It is, therefore, possible that IL-1-induced NF-κB DNA-binding activity might involve activation of protein tyrosine kinase. To test this hypothesis, we have measured protein tyrosine kinase activity following treatment of cells with IL-1α and examined the IL-1α-induced NF-κB in the presence of a protein tyrosine kinase inhibitor, herbimycin A [14–17]. In vitro, herbimycin A inhibits the tyrosine kinase activity of *v-src* [16]. In vivo, herbimycin A reduces phosphorylation of proteins on tyrosine residues [17]. The effect of herbimycin A appears to be specific since it reverses cell transformation induced by tyrosine kinase oncogenes but has no effect on transformation induced by other kinase oncogenes [15,16].

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2. MATERIALS AND METHODS

2.1. *Cell lines*

The 70Z/3 cell line was derived from a nitrosourea induced tumor as described previously [18]. Our 70Z/3 cells came from Drs. Kincade and Paige [18]. EL-4 6.1 C10 is a variant subclone derived from EL-4 thymoma and came to us from Drs. Lowenthal and MacDonald [3]. Cells were grown in complete RPMI 1640 medium supplemented with 5% FCS, 2 mM glutamine, 50 μM mercaptoethanol, penicillin (100 units/ml), and streptomycin (0.01%) at 37°C and humidified with a 7%:93% CO_2/air gas mixture.

2.2. *Reagents*

Recombinant human IL-1α was a gift from Dr. S.K. Dower (Immunex Corp., Seattle, WA). Herbimycin A was isolated as described previously [15]. RPMI 1640 media and Protein Tyrosine Kinase Assay Kit was purchased from Gibco-BRL (Gaithersburg, MD).

2.3. *Cell extracts*

10^7 cells were washed once with 1 ml PBS and once with 1 ml of lysis buffer (10 mM HEPES, 10 mM KCl, and 1.5 mM $MgCl_2$, pH 7.9, 4°C). Cells were lysed by suspending the cell pellet in 20 μl of lysis buffer containing 0.1% NP-40 for 10 min on ice. The soluble fraction of cell lysates was separated from the particulate fraction by microcentrifugation (5 min at 12,000 × g, 4°C). The supernatant representing the soluble fraction of the cell lysates was saved (−70°C). The pellet consisted mostly of intact nuclei. Nuclear proteins were extracted as described previously by Dignam [19] and were saved (−70°C) for electrophoretic mobility shift assay (EMSA).

2.4. *Protein tyrosine kinase activity measurements*

Tyrosine kinase activity in the soluble fraction of the cell lysate was assayed using the RR-SRC assay system as per manufacturers protocol (Protein Tyrosine Kinase Assay System, Gibco-BRL, Gaithersburg, MD). Briefly, aliquots containing equal amount of protein were incubated with or without substrate peptide in the presence of [γ-^{32}P]ATP (30°C). Protein was TCA-precipitated and incubated with a phosphocellulose disc. After extensive wash ^{32}P counts were measured using a scintillation counter. Counts representing RR-SRC phosphorylation were calculated as the difference between counts with or without RR-SRC peptide.

2.5. *Electrophoretic mobility shift assay (EMSA)*

The NF-κB probe was a synthetic double-stranded oligonucleotide with the sequence 5'TGACAGAGGGGACTTTCCGAGAGGA3'. The oligonucleotide probe was end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The binding reaction of the DNA probe with nuclear protein extracts and polyacrylamide gel electrophoresis were performed as described previously [1].

3. RESULTS AND DISCUSSION

To test a hypothesis that IL-1α activates NF-κB DNA-binding activity by activating protein tyrosine kinase, we first examined whether IL-1α stimulates protein tyrosine kinase activity. 70Z/3 or EL-4 6.1 C10 cells were treated with IL-1α and at given time points 10^7 cell aliquots were harvested and protein tyrosine kinase activity in the soluble fraction of the cell lysates was assayed using the RR-SRC peptide substrate (RR-LIEDAEYAARG). This peptide is derived from the amino acid sequence surrounding the phosphorylation site in pp60^{src} and is specific for protein tyrosine kinase [20]. Fig. 1 illustrates results from these experiments. In both cell lines IL-1α induced a transient increase in protein tyrosine activity; first detected at 2 min, it peaked at 5 min and was down at 15 min. Activation of cells through a number of known receptors involves activation of protein tyrosine kinases [21–23]. Some receptors, like the insulin, PDGF or EGF receptors are tyrosine kinases, while others like the IL-2 receptor or the surface immunoglobulin increase tyrosine phosphorylation even though they are not tyrosine kinases [24,25]. EL-4 6.1 C10 express type I IL-1 receptor that is structurally different from the type II IL-1 receptor expressed on 70Z/3 cells [26,27]. Neither type I nor type II IL-1 receptor is a tyrosine kinase [26,27], but as in the case of the IL-2 receptor or the surface immunoglobulin our study shows that IL-1 receptors can nonetheless stimulate protein tyrosine kinase activity. It remains to be determined whether or not the same protein tyrosine kinase(s) are activated by IL-1α in 70Z/3 and EL-4 6.1 C10 cells. As in the case of the lymphoid cell lines used in this study, activation of protein tyrosine kinase activ-

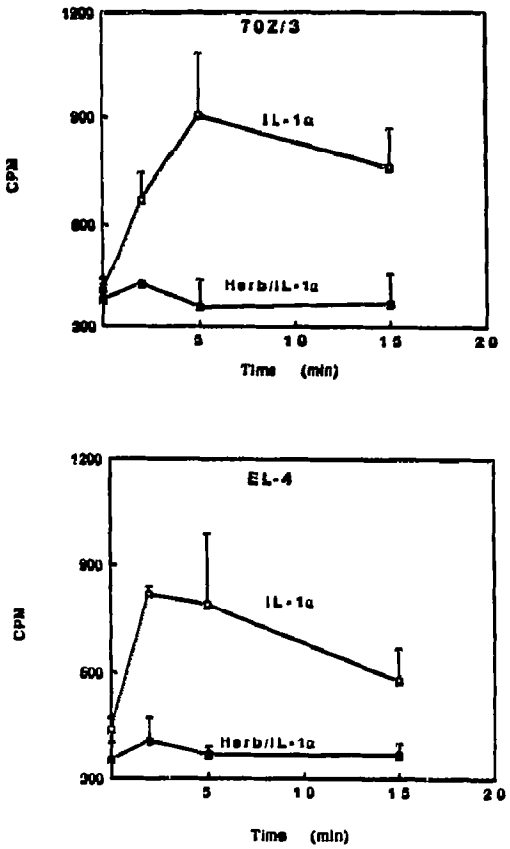

Fig. 1. Time course of protein tyrosine kinase activity in cell lysates from 70Z/3 and EL-4 6.1 C10 cells after IL-1α treatment. Cells at 10^6 cells/ml in RPMI 1640/5% FCS were pre-treated with or without one hour of herbimycin A (20 μg/ml) pulse. After a wash, cells were resuspended in fresh RPMI 1640/5% FCS media and were treated with IL-1α; 70Z/3 with 10^{-10} M and EL-4 6.1 C10 with 10^{-12} M. At given time points protein tyrosine kinase activity in the soluble fractions of the cell lysates was assayed as per the manufacturers protocol (Protein Tyrosine Kinase Assay System, Gibco-BRL). Results are represented as means ± SE of at least four different experiments done in duplicates.

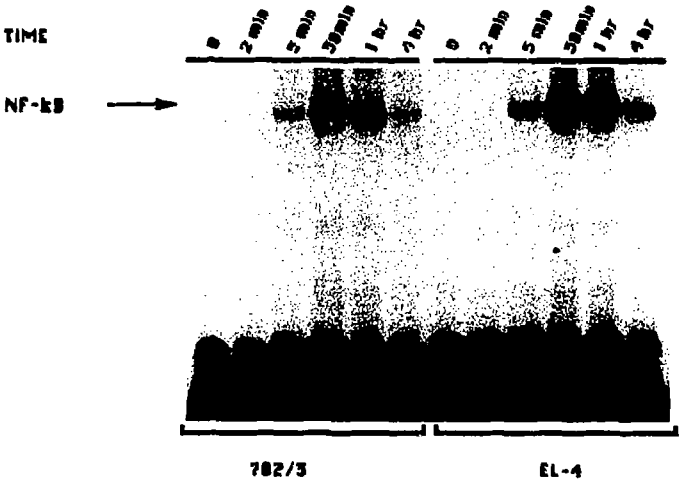

Fig. 2. Time course of NF-κB DNA-binding activity after IL-1α treatment of 70Z/3 and EL-4 6.1 C10 cells. Cells were treated as described in Fig. 1. At the given time points nuclear proteins were extracted from 10×10^6 cells. NF-κB DNA-binding activity in nuclear extracts was assayed by electrophoretic mobility shift assay (EMSA) using the κB double-stranded synthetic oligonucleotide [1].

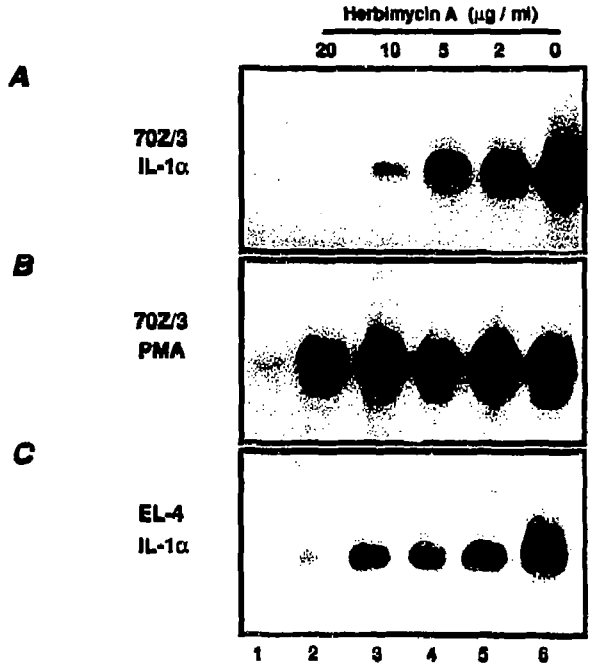

Fig. 3. EMSA of NF-κB after IL-1α or PMA treatment in cells pretreated with various concentrations of herbimycin A pulse. Cells were pre-treated with (lanes 2–5) or without (lanes 1 and 6) one hour of herbimycin A pulse with the indicated concentrations. After a wash cells were resuspended in fresh RPMI 1640/5% FCS media and were treated for 30 min (lanes 2–6) with either 10^{-10} M IL-1α (70Z/3 cells; panel A), 10^{-7} M PMA (70Z/3 cells; panel B) or with 10^{-12} M IL-1α (EL-4 6.1 C10; panel C). NF-κB was assayed by EMSA as in Fig. 2.

ity by IL-1 has also been recently reported in fibroblasts [28].

In 70Z/3 and EL-4 6.1 C10 cells IL-1α activates NF-κB DNA-binding activity [8]. Next we wished to determine the kinetics of IL-1α-induced NF-κB and compare it to the time course of IL-1α-induced protein tyrosine kinase activity. After treatment with IL-1α, cells were harvested, nuclei isolated and levels of NF-κB in nuclear extracts were assayed by EMSA using a synthetic oligonucleotide containing the κB enhancer element (GGGGACTTTCC) from the κ light chain immunoglobulin gene [2]. Results from these experiments are shown in Fig. 2. As was the case with induction of protein tyrosine kinase activity, IL-1α induced transient activation of NF-κB DNA-binding activity. DNA-binding activity of NF-κB was first detected 5 min following stimulation with IL-1α, peaked at 30 min and was nearly down to baseline at 4 h. Comparison of Figs. 1 and 2 shows that IL-1α-enhanced protein tyrosine kinase activity appears to precede the activation of NF-κB.

To test whether protein tyrosine kinase might be involved in the IL-1-induced NF-κB DNA-binding activity we used the irreversible protein tyrosine kinase inhibitor herbimycin A [14–17]. Since herbimycin A is an irreversible inhibitor of protein tyrosine kinases we reasoned that pre-treating cells with a pulse of this drug will allow us to use higher concentration of this drug while maintaining good cell viability. Accordingly, cells were pre-treated with various concentrations of herbimycin A and after one hour of treatment cells were washed, resuspended in fresh media and were then treated with IL-1α. After 30 min treatment (time at which IL-1α induces maximal NF-κB response (Fig. 2)), cells were harvested and nuclear extracts were assayed for NF-κB activity by EMSA. Results from these experiments are shown in Fig. 3. Pre-treatment of 70Z/3 cells with 2 μg/ml herbimycin A significantly reduced the ability of IL-1α to activate NF-κB and at 20 μg/ml the IL-1α-induced NF-κB was completely blocked (Fig. 3A). The effect of herbimycin A on IL-1α-induced NF-κB in EL-4 6.1 C10 cells was similar (Fig. 3C).

Previous results from this and other laboratories have shown that the mechanism of activation of NF-κB by PMA may be different from the mechanisms used by physiological inducers such as IL-1 or TNF [8,9]. Therefore, we next tested the effects of herbimycin A on PMA-induced activation of NF-κB in 70Z/3 cells. We have previously shown that PMA is an effective activator of NF-κB and protein kinase C in 70Z/3 cells [8,10]. As before, cells were pre-treated with or without a one hour herbimycin A pulse and after a wash cells were incubated with 10^{-7} M PMA. After 30 min of treatment with PMA, nuclear extracts were assayed for NF-κB using the κB probe and EMSA. Gels from this experiment are shown in Fig. 3B. These results showed that in a sharp contrast to the IL-1α results, herbimycin A did not block the PMA-induced activation of NF-κB. The effects of PMA and IL-1α in herbimycin A-treated EL-4 6.1 C10 cells could not be compared because PMA

does not activate NF-κB in these cells [8]. Similarly to these results, a differential effect of herbimycin A has previously been reported for the T-cell receptor- and PMA-mediated IL-2 production and IL-2 receptor expression in human peripheral T cells [29].

To show that herbimycin A blocks stimulation of tyrosine kinase(s) we assayed protein tyrosine kinase activity in cell lysates derived from IL-1α- and herbimycin A-treated cells. As illustrated in Fig. 1, pre-treatment of 70Z/3 and EL-4 6.1 C10 cells with a 20 μg/ml herbimycin A pulse blocked the IL-1α-enhanced protein tyrosine kinase activity.

Our studies demonstrate differential effects of herbimycin A on the induction of NF-κB in response to IL-1α and PMA. These observations indicate that the two inducing agents activate NF-κB DNA-binding activity by different pathways. While in the case of PMA there is a strong evidence for a protein kinase C-dependent pathway [7–9], the IL-1 pathway appears to be independent of the known isoforms of protein kinase C [8]. This is in agreement with studies from other laboratories which showed that TNF [9] and the phosphatase inhibitor, okadaic acid [30], activate NF-κB DNA-binding activity independent of protein kinase C. Although all the cellular effects of herbimycin A are not yet known, this and a number of other studies suggest that the inhibitory effect is specific for protein tyrosine kinases [14,15]. Thus, the results from the present study point to a protein tyrosine kinase-dependent event transducing the IL-1α-induced NF-κB DNA-binding activity.

Although it now appears that IL-1α and PMA activate NF-κB by different pathways, it remains to be established whether or not these pathways overlap and at which point they may converge. It is conceivable that the two pathways converge at an early step in the cascade of events leading to the activation of NF-κB. In fact, it cannot be ruled out that the shared portion of such a pathway, if it exists, may include a protein tyrosine kinase and/or yet unidentified isoform of protein kinase C. On the other hand, it may turn out that the IL-1α and the PMA pathways do not overlap until they both terminate by phosphorylating different sites of IκB. A gene encoding IκB-like activity has recently been cloned [31]. The predicted protein sequence contains a consensus sequence for tyrosine and protein kinase C phosphorylation sites. It is possible that IκB could dissociate from NF-κB by phosphorylation of either one of the IκB phosphorylation sites. This would be consistent with the hypothesis that PMA activates NF-κB DNA-binding activity by stimulating protein kinase C which phosphorylates IκB on the serine residue, while IL-1α activates NF-κB by stimulation of protein tyrosine kinase which phosphorylates IκB on the tyrosine residue. Phosphoamino acid analysis of in vivo ^{32}P-labelled IκB following PMA and IL-1α treatments will be required to test this hypothesis.

It appears that the phosphorylation-induced dissociation of IκB from NF-κB and the resulting activation of DNA-binding [5–7] might be only one of many components of a complicated network of events and factors regulating gene expression from the κB enhancer element. For example, a number of constitutive and inducible κB-binding proteins have recently been described. Most but not all appear to be homologous to *rel* [32,33]. Furthermore, we have recently identified a constitutive 65 kDa κB-binding phosphoprotein that is closely associated with an inducible serine/threonine kinase(s) [34]. It remains to be defined how the 65 kDa κB-binding phosphoprotein and its associated kinase activity is structurally and functionally related to the other constitutive and inducible κB-binding complexes.

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