Volume 319, number 1,2, 100–104 FEBS 12201 March 1993

Tyrosine kinase activity is involved in the protein kinase C induced expression of interleukin-1β gene in monocytic cells

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Received 29 December 1992

The role of protein tyrosine kinases in the expression of interleukin-1β (IL-1β) gene in response to phorbol esters (PMA) in THP-1 cell line was investigated. Genistein, a specific tyrosine kinase inhibitor, inhibited PMA induced IL-1β protein and mRNA levels in THP-1 cells. Genistein did not have a significant effect on PMA induced activity in transient transfection assays using reporter gene constructs containing the PMA responsive sequence of the IL-1β gene linked to IL-1β promoter, or five repeats of PMA responsive sites (AP-1 sites) in front of a thymidine kinase promoter. This indicates that the tyrosine kinase activity required for the PMA induced IL-1β expression is coupled downstream from or functions independent of the PMA induced AP-1 activity.

Interleukin-1β; THP-1 cell line; Phorbol ester; Tyrosine kinase; Protein kinase C; AP-1

1. INTRODUCTION

Interleukin-1 (IL-1) is a cytokine mainly produced by cells of the monocyte/macrophage lineage (reviewed in [1]). Two distinct molecular forms, IL-1α and IL-1β, have been characterized and their genes cloned and sequenced [2]. These molecules possess many biological activities in immune and inflammatory responses, and in normal hematopoiesis. Mature monocyte/macrophages do not normally express IL-1 genes, but their expression can be induced by a variety of activators [3].

The signal transduction pathways leading to the expression of IL-1β gene in monocytes/macrophages have been partially characterized. Activation of protein kinase C (PKC) (a serine-threonine kinase) has been reported to play an important role in the induction of IL-1β expression in different cell types and after different stimuli [4,5]. PKC regulates transcription by activating different transcription factors, which bind to specific *cis*-elements on PKC activated genes (reviewed in [6]). The best characterized PKC dependent pathway is mediated by the fos/jun transcription factor complex (activating protein 1; AP-1 complex), which binds to the phorbol ester response element (AP-1 binding site) on PKC inducible genes [7]. AP-1 is composed of heterodimers of the *jun* and *fos* proto-oncogene families or homodimers of the *jun* family. AP-1 activity is regulated both at the level of transcription as well as by post-translational modifications of pre-existing AP-1 [6]. It has recently been demonstrated that a 180 base pair long enhancer sequence in the IL-1β gene, which is required for the phorbol ester induced IL-1β expression, contains a putative AP-1 binding site [8].

Another major protein kinase family, tyrosine kinases, is involved in the signal transduction of many receptor systems and in the proliferation and differentiation of normal and malignant cells (for a review see [9]). Tyrosine kinases can be divided into two groups; to those expressing extracellular ligand binding domains and an intracellular tyrosine kinase domain (e.g. receptors for various polypeptide growth factors like PDGF receptor) and to those residing entirely intracellularly (e.g. members of the *src*, *fes/fps* and *abl* gene families). The increase in general tyrosine kinase activity [10] and in the expression of certain members of *src* and *fes* family genes during myeloid differentiation and monocyte activation [11–13] first suggested that tyrosine kinases also have a role in mediating activation signals in differentiated monocytes. Increased protein phosphorylation on tyrosine in response to bacterial LPS was recently reported in monocyte/macrophages [14]. A role for tyrosine kinases in the regulation of IL-1 expression has also been demonstrated. Granulocyte-macrophage colony stimulating factor (GM-CSF), which activates protein tyrosine phosphorylation, induces IL-1 production in human mononuclear cells [15], and IL-1β expression induced via MHC Class II molecules is inhibited by tyrosine kinase inhibitors in THP-1 cells [16] and in human monocytes (Palkama and Hurme, Hum. Immunol., in press).

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Abbreviations: PMA, phorbol 12-myristate-13-acetate; PKC, protein kinase C; AP-1, activating protein 1; CAT, chloramphenicol acetyl transferase.

In addition to direct activation of PKC, protein phosphorylation on tyrosine has been reported after phorbol ester stimulation in various cell types [17–19]. In this work we demonstrate that tyrosine kinase activity is involved in phorbol ester induced IL-1β expression in the monocytic cell line THP-1. The coupling of the tyrosine kinase activity involved in phorbol ester induced IL-1β expression to phorbol ester induced AP-1 enhancer activity, was also investigated.

2. MATERIALS AND METHODS

2.1. *Reagents*

Phorbol 12-myristate-13-acetate (PMA) was purchased from Sigma Chemical Co. (St. Louis, MO). Protein kinase C inhibitor H7 (1-[5-isoquinolonesulfonyl]-2-methylpiperazine dihydrochloride) and protein kinase A (PKA) inhibitor HA 1004 (*N*-(2-guanidinoethyl)-5-isoquinoline-sulfonamide hydrochloride) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Genistein was purchased from Gibco Research Products Life Technologies Inc. (Gaithersburg, MD).

2.2. *Cell cultures*

The THP-1 cell line was obtained from The American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium (Flow Laboratories, Irvine, Renfrewshire, UK) containing 10 mM HEPES, 2 mM L-glutamine, 70 μM 2-mercaptoethanol, antibiotics and 10% fetal calf serum (Flow). The cells were tested monthly for Mycoplasma and were found to be negative.

2.3. *IL-1β protein induction and analysis*

During the exponential growth phase, THP-1 cells were cultured with the indicated stimulators at 10^6 cells/ml in 24-well plates (Costar, Cambridge, MA). After 24 h cells were collected with the supernatants to determine total (intracellular, cell-associated and secreted) IL-1β produced. Cells were disrupted with three cycles of freezing and thawing and all samples were stored at −20°C. IL-1β contents of the samples was measured by an IL-1β ELISA (Cistron, Pine Brook, NJ) according to the manufacturer's instructions.

2.4. *RNA isolation and analysis*

0.5×10^6 THP-1 cells/ml were cultured in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA) and stimulated with the indicated stimulators. After 6 h cells were harvested and total cellular RNA was isolated by guanidium isothiocyanate lysis and CsCl centrifugation [20,21]. The RNA isolated was quantitated spectrophotometrically and 30 μg samples were size-fractionated on 1.2% agarose-formaldehyde gels, transferred to a nylon membrane (Pall, Glen Cove, NY), dried and baked at 80°C. The IL-1β cDNA probe (HU-IL-1β, pcDSRa) used was provided by Dr. Kari Varkila (DNAX Research Institute, Palo Alto, CA). The RNA levels on the nylon membranes were also quantitated by using a constant probe, glyceraldehyde phosphate dehydrogenase (pRGAPDH-13), which was a gift from Dr. Kari Alitalo (Dept. Pathology, University of Helsinki). The IL-1β cDNA insert (*Bam*HI-digested fragments from the HU-IL-1β plasmid) and the GAPDH insert (*Pst*I-digested fragments from pRGAPDH-13) were labeled with ^{32}P using a random-primed DNA labeling kit purchased from Boehringer-Mannheim GmbH, (Mannheim, Germany). Prehybridizations and hybridizations were performed in a solution containing 50% formamide, 5× Denhardt's solution, 5× SSPE and 0.5% SDS. Filters were washed in 1× SSC plus 0.1% SDS, twice for 30 min at room temperature and once at 60°C for 30 min. Subsequently, the filters were exposed to Kodak AR X-Omat films at −70°C with intensifying screens.

2.5. *Transfections*

DNA was introduced into THP-1 cells by electroporation as described by Pahl et al. [22]. Briefly, the medium was changed and the cells were grown at the density of 0.2×10^6/ml. 16–24 h later the cells were washed twice with RPMI-1640 and resuspended at 28×10^6/ml in RPMI-1640. 20 μg of plasmid DNA in 50 μl RPMI-1640 was mixed with 0.5 ml of cell suspension and placed into a 0.4 cm electroporation cuvette. The cells were incubated 5 min at room temperature prior to electroporation at 960 μF and 300 V (Bio-Rad Laboratories, Richmond, VA).

After electroporation the cuvettes were transferred into an ice bath for 5 min. After transfection the cells were cultured at 10^6/ml with the indicated stimulators. Two different CAT constructs were used (Fig. 1). Col-TREx5/TK-CAT [7], which contains five copies of the AP-1 binding site from the human collagenase gene, the thymidine kinase promoter of *Herpes simplex* virus and the chloramphenicol acetyl transferase (CAT) reporter gene, was a generous gift of Dr. M. Karin (Department of Pharmacology, University of California, San Diego, CA). The IL-1β-X-CAT was a gift from Dr. G. Bensi (Sclavo Research Centre, Siena, Italy). It contains two 234 bp repeats of the phorbol ester responsive IL-1β enhancer located between positions −2982 and −2748 linked to IL-1β promoter from 132 bp immediately upstream of the transcriptional start site to +548 of the second exon of the IL-1β gene, SV-40 promoter and enhancer and CAT gene [8]

2.6. *Assay of CAT activity*

24 h after stimulation, the cells were harvested to CAT assay. The cells were washed twice in PBS and resuspended in 0.25 M Tris-HCl, pH 7.4. Cell extracts were prepared by four cycles of freezing and thawing. The nonchromatographic CAT assay was performed as previously described [23]. Incubation mixture contained 20 μl of 8 mM chloramphenicol, cell extract (30 μg), 20 μl of [^{14}C]acetyl coenzyme A (5 μCi/ml), 0.25 M Tris-HCl, pH 7.4, to bring the reaction volume to 0.1 ml. Solid [^{14}C]acetyl coenzyme A (Amersham) was dissolved in water (50 μCi/ml) and diluted 1:10 with 0.5 mM unlabeled enzyme (Boehringer-Mannheim) before use. Incubation was performed at 37°C for 1 h. The reaction was stopped with 0.6 ml of cold ethyl acetate, which was also used to extract the chloramphenicol. The organic layer was mixed with 3.0 ml of Optiscint Hisafe (Wallac, Turku, Finland) and radioactivity was determined by liquid scintillation β-counter. Under these incubation conditions the reaction was at the linear range as determined with purified CAT enzyme (Promega Corp., Madison, WI).

3. RESULTS

3.1. *The effect of genistein, an inhibitor of protein tyrosine kinases, on IL-1β production in THP-1 cells*

We examined the possible role of tyrosine kinase activity in the regulation of IL-1β protein production in response to phorbol esters in THP-1 cells. THP-1 cell line is derived from a patient with monocytic leukemia [24] and it resembles normal monocytes in its ability to produce IL-1 in response to various stimulators (e.g. LPS and phorbol esters [25]). THP-1 cells were preincubated for 30 min with different concentrations of genistein, a specific inhibitor of tyrosine protein kinase activity of both receptor type- (e.g. EGF-receptor) and intracellular (e.g. $pp60^{v\text{-}src}$ tyrosine kinases [26], before leaving the cells unstimulated or stimulating them with the optimal concentration (10 ng/ml) of PMA for 24 h. After that cultures were harvested and analyzed for their IL-1β protein content in an IL-1β specific ELISA as described in section 2. Genistein concentration dependently inhibited PMA induced IL-1β production (Table I).

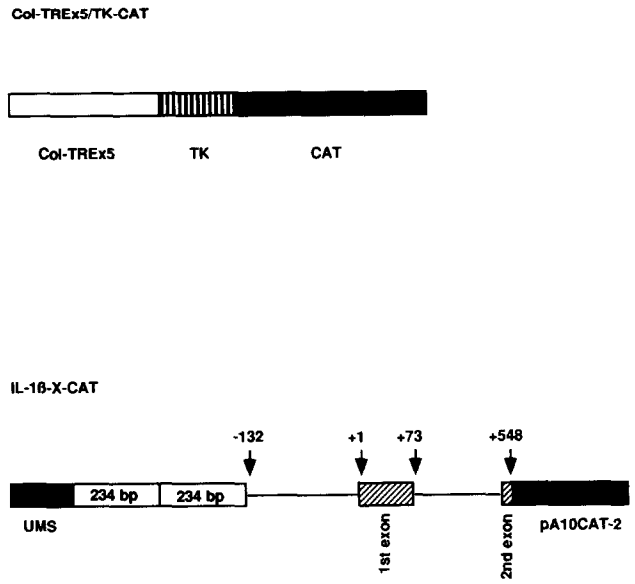

Fig. 1. Diagrammatic representation of the plasmids used. Col-TREx5/TK-CAT: five copies of the PMA responsive elements of the human collagenase gene (AP-1 binding sites) are linked to the pBLCAT-2 plasmid containing the *Herpes simplex* virus thymidine kinase promoter and CAT coding gene. IL-1β-X-CAT: two copies of the PMA responsive sequence of the IL-1β gene between sequences −2,982 and −2,748 (234 bp boxes) are linked to the IL-1β promoter (from −132 to +548 of the second exon) and pA10CAT-2 plasmid containing the SV40 promoter and enhancer and CAT coding gene. Stippled box: the upstream sequence of the murine c-*mos* gene (UMS) used as the transcription terminator.

3.2. *The effect of tyrosine kinase inhibition on phorbol ester mediated IL-1β RNA expression in THP-1 cell line*

We then investigated, whether the tyrosine kinase activity involved in IL-1β expression could also be seen at the mRNA level. THP-1 cells were preincubated with the indicated concentrations of genistein for 30 min and then stimulated with 10 ng/ml PMA for 6 h. Cells were then harvested and total cellular RNA was isolated and analyzed for the expression of IL-1β mRNA. Again, genistein dose dependently inhibited IL-1β mRNA levels induced by PMA in THP-1 cells (Fig. 2).

3.3. *Genistein does not modulate the PMA-induced AP-1 enhancer activity*

To localize the site of action of the tyrosine kinase activity involved in IL-1β expression, the effect of genistein in THP-1 cells transiently transfected with Col-TREx5/TK-CAT, a reporter gene construct containing 5 repeats of the TRE-element upstream of the *Herpes simplex* virus-thymidine kinase-promoter CAT gene (Fig. 1). As expected, PMA strongly elevated AP-1 enhancer activity, the increase was 6.9-fold when compared to unstimulated cells in 4 independent experiments (Fig. 3A). 30 μg/ml genistein did not have a significant inhibitory effect on the PMA induced AP-1 enhancer activity. The serine-threonine kinase inhibitors H7 and HA 1004 were used as controls. 25 μM H7, a preferential PKC inhibitor, completely inhibited PMA induced AP-1 enhancer activity, while the same concentration of HA 1004, a preferential protein kinase A inhibitor with a weak PKC inhibitory effect, partially inhibited PMA induced AP-1 activity. The serine-threonine kinase inhibitors used clearly inhibited AP-1 activity, while genistein at concentrations which effectively inhibited IL-1β expression, remained uneffective. This indicates that the inhibitory effect of genistein on IL-1β expression is not mediated via unspecific inhibition of serine-threonine kinases.

To determine the effect of genistein on the reporter gene construct containing the phorbol ester responsive sequences of the IL-1β gene, we transfected THP-1 cells with IL-1β-X-CAT, which contains two repeats of the phorbol ester responsive IL-1β enhancer linked to IL-1β promoter (Fig. 1) [8]. 10 ng/ml PMA induced a ca. 4-fold increase in CAT activity of IL-1β-X-CAT transfected cells, which again was not inhibited by 30 μg/ml genistein (Fig. 3B).

4. DISCUSSION

In this study we demonstrate that tyrosine kinases functioning downstream from or independent of PKC dependent AP-1 activation are involved in the phorbol ester mediated IL-1β expression in monocytic cells.

Activation of PKC is known to play an important role in phorbol ester induced IL-1β expression [4,5]. In addition to directly activating PKC [27], phorbol esters have been shown to induce protein phosphorylation on tyrosine in various cell types [11–13]. We have also demonstrated that PMA induced protein phosphorylation on tyrosine in THP-1 cells, when determined with antiphosphotyrosine immunoblotting of whole cell lysates (results not shown). To investigate the role of tyrosine

Table I

The effect of genistein, a tyrosine kinase inhibitor, on interleukin-1β (IL-1β) production by THP-1 cells

Stimulators	IL-1β (pg/ml)[a]	%[b]
None	<20	
PMA 10 ng/ml	3,398	100
PMA 10 ng/ml + genistein 30 μg/ml	625	25 ± 12
PMA 10 ng/ml + genistein 20 μg/ml	1,934	64 ± 28
PMA 10 ng/ml + genistein 10 μg/ml	3,423	121 ± 33

THP-1 cells (10^6 cells/ml) were preincubated for 30 min with the indicated concentrations of genistein and then stimulated with PMA. After 24 h the cultures were harvested and their IL-1β content was measured by IL-1β specific ELISA assay.

[a] The data is shown from a single experiment.
[b] Mean percent of IL-1β production (±S.D.), when compared to PMA stimulation, of four independent experiments.

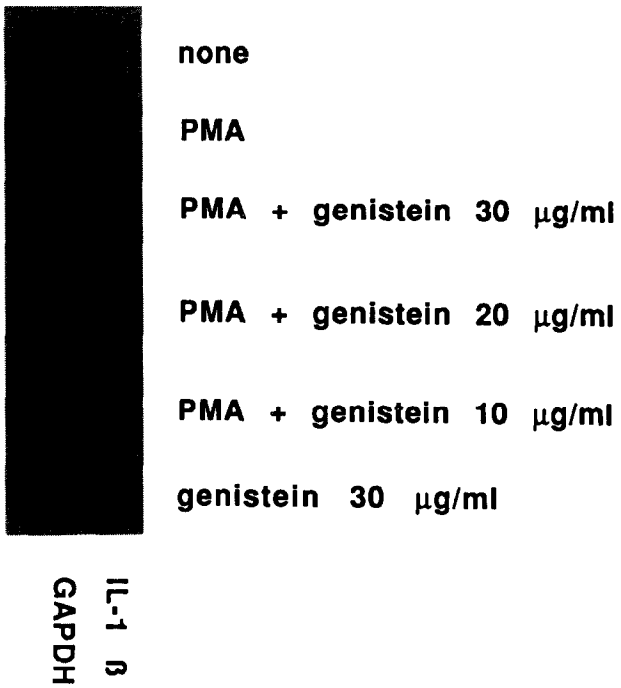

Fig. 2. The effect of genistein on phorbol ester mediated IL-1β expression in THP-1 cells. 0.5×10^6 cells/ml THP-1 cells were cultured with media alone or with the indicated concentrations of the tyrosine kinase inhibitor genistein for 30 min. After that cells were left unstimulated or stimulated with 10 ng/ml PMA. After 6 h cells were harvested and total cellular RNA was isolated and analyzed for IL-1β expression. The experiment was repeated three times with identical results.

kinase activation in IL-1β expression, the effect of genistein, a specific tyrosine kinase inhibitor in concentrations up to 40 μg/ml [26], on phorbol ester induced IL-1β production was determined. Genistein concentration dependently inhibited PMA induced IL-1β protein production and IL-1β RNA expression in THP-1 cells, indicating that protein tyrosine kinases are involved in phorbol ester mediated IL-1β expression.

PKC and tyrosine kinase signal transduction pathways are known to interact in several cellular systems. Attenuation of receptor tyrosine kinase activity by PKC has been demonstrated in EGF-receptor [28]. In T lymphocytes tyrosine phosphorylation is required for phospholipase Cγ mediated inositol phospholipid hydrolysis and subsequent PKC activation in T-cell receptor mediated activation. Members of the *src* family, $p59^{fyn}$ associated to the TCR/CD3 complex and $p56^{lck}$ associated to the CD4/CD8 molecule are the likely candidates to mediate this tyrosine phosphorylation signal (reviewed in [29]). A similar tyrosine kinase mediated PKC activation cascade has been demonstrated in signalling via MHC class II molecules in lymphocytes [30]. Data demonstrating PKC mediated tyrosine kinase activation also exists, since tyrosine phosphorylation of lymphoid microtubule associated protein-2 kinase (MAP-2 kinase) is dependent on PKC [31,32].

It has recently been shown that an inducible enhancer sequence, which contains a putative AP-1 binding site, is required for IL-1β expression in response to phorbol esters in myeloid cells [8]. Activation of AP-1 via PKC is well characterized [6]. We wanted to determine, whether the tyrosine kinase involved in phorbol ester induced IL-1β expression is coupled to phorbol ester mediated AP-1 activation. The effect of genistein in phorbol ester induced AP-1 activity was investigated using two different reporter gene constructs; IL-1β-X-CAT, containing the phorbol ester responsive sequence of IL-1β gene coupled to IL-1β promoter and CAT gene, and Col-TREx5/TK-CAT, which contains 5 repeats of AP-1 binding sites from human collagenase gene in front of the thymidine kinase promoter and CAT gene. The same concentration of genistein (30 μg/ml), which inhibited IL-1β expression in THP-1 cells, did not have a significant inhibitory effect on phorbol ester induced CAT activity in either reporter gene construct. The serine-threonine kinase inhibitors H7 and HA 1004, used as specificity controls for genistein,

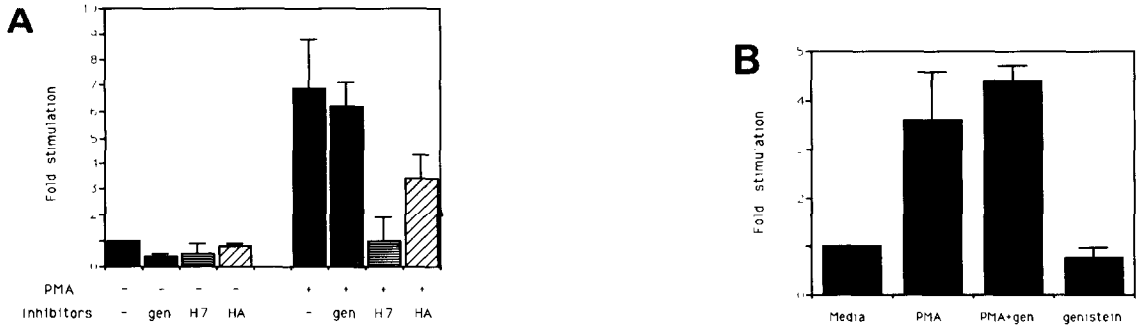

Fig. 3. The effect of genistein on PMA induced AP-1 enhancer activity in THP-1 cells. (A) THP-1 cells were transfected with the Col-TREx5/TK-CAT plasmid. Cells were either left unstimulated or cultured for 30 min with the indicated inhibitors: 25 μM H7, 25 μM HA 1004 or 30 μg/ml genistein. After transfections cells were either left unstimulated or stimulated with 10 ng/ml PMA. After 24 h cells were lysed and CAT activity determined. The data shown are expressed as mean fold of increase (+S.D.) when compared to unstimulated cells of 4 independent experiments. (B) THP-1 cells were transfected with IL-1β-X-CAT plasmid. After transfections cells were cultured with no stimulators or 30 μg/ml genistein for 30 min. After that cells were either left unstimulated or cultured with 10 ng/ml PMA for 24 h and CAT activity was determined. The data shown are expressed as mean fold of increase (+S.D.) when compared to unstimulated cells of 4 independent experiments.

clearly inhibited PMA induced AP-1 CAT activity. Our data indicates that genistein inhibitable tyrosine kinases are not involved in phorbol ester induced AP-1 enhancer activity.

Little data on the role of tyrosine kinases in the regulation of transcriptional activation exists. Recently the activation of the transcription factor interferon-α-stimulated gene factor 3 (ISGF3) via tyrosine phosphorylation by interferon-α and -γ was reported [33]. This is the first direct evidence of tyrosine kinases activating transcription factors. The transcriptional activation of IL-1β expression is still poorly characterized. In addition to the phorbol ester responsive element located between positions −2,982 and −2,795 upstream of the IL-1β transcriptional start site, elements located in the IL-1β gene enhancer/promoter regions have been reported to regulate both the constitutive and inducible IL-1β expression in monocytic cells [8,34]. However, there has been no evidence on the role of tyrosine kinases in this regulation. We have preliminary data showing that genistein strongly reduces the constitutive expression of a reporter gene construct containing the region between −1,100 and +11 base pairs of the IL-1β gene, thus suggesting that constitutive, rather than PMA inducible tyrosine kinases are required for the IL-1β expression. We are currently trying to localize the target sequence of the tyrosine kinase activity within this region.

Acknowledgements This work was supported by grants from the Finnish Cultural Foundation, the Paulo Foundation, the Finnish Cancer Society, the Finnish Medical Foundation Duodecim and Leiras Medical Corporation.

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