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Calcium ionophore A23187 induces interleukin-8 gene expression and protein secretion in human monocytic cells

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The regulation of the expression of the interleukin-8 (IL-8) gene in human monocytic cell lines has been investigated. Agents such as interleukin-1 (IL-1) or interferon-γ (IFNγ) did not induce increased IL-8 expression in THP-1 or U937 cells. Bacterial lipopolysaccharide endotoxin (LPS) or phorbol myristate acetate (PMA) alone induced suboptimal expression as assessed by Northern blotting; however, preincubation of cells with PMA followed by endotoxin induced much higher levels of IL-8 mRNA. Incubation of the cells with the calcium ionophore A23187 resulted in consistent increased IL-8 gene expression comparable to that of cells treated with endotoxin alone. In addition to inducing IL-8 mRNA this calcium ionophore also induced IL-8 protein synthesis as assessed by immunofluorescence and secretion as detected by ELISA. These results indicate that increases in intracellular calcium result in IL-8 gene expression and protein secretion.

Interleukin-8; Gene expression; Monocyte

1. INTRODUCTION

In recent years a family of small molecular weight peptides, characterized by C-C or C-X-C motifs, has been identified the members of which are potent chemoattractants for neutrophil leukocytes, monocytes or T lymphocytes [1]. One of the best characterised members of this chemokine family is interleukin-8 (IL-8), a protein secreted from a wide range of cell types which is a potent chemottractant for polymorphonuclear leucocytes (PMNs). In addition to being a chemotactic molecule this protein also primes for subsequent responses to several natural and artifactual agonists [2,3]. IL-8 was first identified as a monocyte-derived neutrophil chemotactic factor and it seems likely that in vivo monocytes are a major source of IL-8 production. Monocytes play a central role in the generation of cytokine-mediated inflammatory events, and it is now well established that proinflammatory factors such as IL-1, tumour necrosis factor (TNF), and LPS induce IL-8 gene expression and protein secretion in primary monocytes. In addition cytokines which downregulate this response have been reported [4–6]. Although the receptors for these monocyte-activating factors have been identified, the intracellular signalling processes leading to increased expression of genes such as IL-8 have yet to be resolved. Genomic sequences upstream of the IL-8 coding region have been identified [7,8], however the mechanism by which the IL-8 gene is regulated is at present unclear. Intracellular calcium has been shown to be an important regulator of many cellular functions, possibly by regulating the activity of calcium- and calmodulin-dependent protein kinases, and calcium and cAMP regulate the transcription of a number of genes [9,10]. Calcium and calmodulin regulate the expression of LPS- induced IL1α, IL-1β and the IL-8-related IP10 gene in murine macrophages [11], therefore it is possible that this cation is important in promoting the expression of other genes such as IL-8. The cell lines THP-1 and U937 have been used as models to investigate signalling in human monocytic cells [12–14], therefore we have used these cells to investigate the induction of IL-8 gene expression. In particular we have investigated the effects of the calcium ionophore A23187 on IL-8 gene expression and we report here that A23187 will induce IL-8 gene expression, protein synthesis and secretion.

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2. MATERIALS AND METHODS

2.1. *Cell culture and treatment*

The human monocytic cell lines THP-1 and U937 were obtained from the American Type Culture Collection and routinely cultured in RPMI 1640 medium (Gibco Ltd) supplemented with 10% foetal calf serum (Gibco Ltd) and penicillin/streptomycin (Gibco Ltd). Log phase cultures at about 2×10^5 cells/ml were treated overnight with 1 μg/ml *Staphylococcus aureus* lipopolysaccharide (LPS, Sigma), 10 nM phorbol myristate acetate (PMA, Sigma) or 1 μM A23187 (Sigma). Some cultures were pretreated for 2 h with PMA before the addition of 1 μg/ml LPS and then cultured overnight. After an 18-h incubation the cells and supernatants were processed for Northern blotting, immunofluorescence or ELISA as described below. In other experiments PMA-treated cells were divided into substrate-adherent and nonadherent populations by washing twice with PBS and harvesting the detached cells which were then processed for RNA extraction.

2.2. *RNA isolation and Northern blotting*

Total cytoplasmic RNA was isolated using guanidinium isothiocya-

nate. 10 μg of total RNA was denatured, separated by electrophoresis through 1% (w/v) agarose gels containing formaldehyde and transferred to Hybond N membranes (Amersham). IL-8 mRNA was detected by hybridisation with a randomly primed probe (Megaprime DNA labelling kit, Amersham) corresponding to the IL-8 coding sequence. The Northern blots were stripped and assessed for equal RNA loading by hybridisation using a β-actin oligonucleotide according to the manufacturer's specifications (Clonetech). Following hybridisation at 65°C, filters were washed to 0.1 × SSC, 0.1% (w/v) SDS at 65°C and exposed to X-ray film at −70°C for 4 days (Hyperfilm, Amersham).

2.3. *Immunofluorescence staining of cells*

Cells were washed three times and fixed by the addition of 3.7% formaldehyde in phosphate-buffered saline (PBS). 100 μl of 0.2% Triton X-100 was added to 100 μl of fixed cells for 10 minutes to permeabilise the cells. After washing three times the cells were stained for IL-8 by the addition of a 1:1000 dilution of a polyclonal anti-IL-8 serum (a kind gift from Dr. S. Kunkel, University of Michigan) followed by biotinylated swine anti-rabbit Ig Fab fragments (Dako) and fluorescein-labelled streptavidin (Dako) with washing between these steps. The cells were washed and mounted in PBS:glycerol (1:9) and viewed using a Zeiss Axioscope microscope equipped with epifluorescence.

2.4. *Quantitation of IL-8 secretion*

Treated cells were centrifuged at 800 × *g* for 10 min to sediment the cells. The supernatants were then centrifuged at 11,000 × *g* for 15 min and the clarified supernatants used for ELISA. The amount of IL-8 secreted into the supernatants was measured using a Quantikine ELISA kit (R&D Systems) exactly as described by the manufacturer

3. RESULTS

3.1. *IL-8 gene expression in human monocytic cell lines*

The effect of a number of agents on IL-8 gene expression in U937 and THP-1 cells was investigated. Untreated cells contained undetectable or barely detectable levels of IL-8 mRNA (Fig. 1, tracks 2,8) whereas treatment with PMA or LPS resulted in readily detectable levels of IL-8 mRNA (Fig. 1, tracks 3,4,9,10). PMA generally induced a slightly greater response than LPS (Fig. 1, tracks 3,4; Fig. 2, tracks 2,3) however occasionally LPS induced the larger response (Fig. 1, tracks 9,10). This variation is not due to cell type but may be due to errors in detection of low-level responses in some experiments. Pretreatment with PMA prior to LPS addition resulted in an augmented IL-8 mRNA response compared to either agent alone (Fig. 1, tracks 5,11). The calcium ionophore A23187 induced readily detectable levels of IL-8 mRNA, quantitatively more than that induced by LPS and comparable with that induced by PMA in both U937 and THP-1 cells (Fig. 1, tracks 6,12; Fig. 2, track 12). Other agents such as IL-1β, IFNγ, platelet activating factor (PAF), formyl-methionyl-leucyl-phenylalanine (fMLP) or leukotriene B_4 (LTB_4) failed to induce IL-8 gene expression (Fig. 2, tracks 7,9,11,13; Fig. 1, track 13), although pretreatment with IFNγ followed by LPS gave a slight response (Fig. 2, track 10). It has recently been reported that adherence of peripheral blood monocytes to plastic induces novel gene expression [16], however separation of the PMA or PMA/LPS-treated cells into adherent and non-adherent

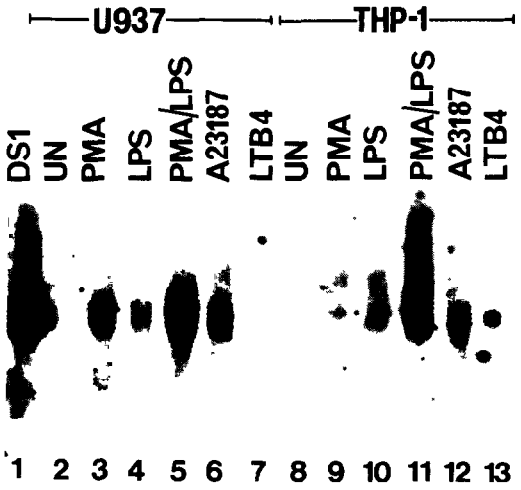

Fig. 1. Expression of IL-8 mRNA in U937 (lanes 2–7) and THP-1 cells (lanes 8–13) stimulated with a variety of agonists. Cells were cultured for 18 h in the presence of: 10 ng/ml PMA (lanes 3 and 9); 1 μg/ml LPS (lane 4 and 10); 10 ng/ml PMA plus 1 μg/ml LPS (lanes 5 and 11); 1 μM A23187 (lanes 6 and 12); 10^{-7} M Leukotriene B_4 (lanes 7 and 13). Lanes 2 and 8 are unstimulated controls and lane 1 is a positive control of DS-1 human fibroblasts stimulated for 4 h with 10 ng/ml IL-1β.

cell showed that both populations contained similar levels of IL-8 mRNA (Fig. 2, tracks 3–6).

3.2. *Immunolocalisation of IL-8 in THP-1 cells*

Although the Northern blotting analysis described above gave important information regarding the steady state levels of IL-8 mRNA in phorbol ester or calcium ionophore-treated cells, it was important to ascertain whether IL-8 protein was synthesised in these cells. Immunofluorescent staining for intracellular IL-8 demonstrated almost undetectable levels in untreated cells (Fig. 3a). Cells which had been treated with both PMA and LPS displayed intense IL-8 staining. This was particularly strong in the perinuclear area of the cell which was probably the golgi and endoplasmic reticulum (Fig. 3d). PMA-treated, LPS-treated and A23187-treated cells all displayed intracellular IL-8 staining, although the intensity of staining was reduced compared with that of PMA plus LPS (Fig. 3b,c,e). Nevertheless these levels were still elevated compared to untreated cells (Fig. 3a) and control background staining using pre-immune serum (Fig. 3f). Cells treated with endotoxin or A23187 typically displayed discrete patches of IL-8 probably corresponding to intracellular vesicles (Fig. 3b,e, arrows).

3.3. *Quantitation of secreted IL-8*

The amount of IL-8 produced by THP-1 cells was measured using a quantitative ELISA system. Untreated THP-1 cells secreted very low levels of IL-8. Cells treated with PMA followed by LPS secreted large amounts of IL-8 and PMA or A23187 alone induced lower levels of IL-8 secretion, although this was still a 25-fold increase over unstimulated cells. LPS induced a

6-fold increase in IL-8 secretion compared with unstimulated cells (Table I).

4. DISCUSSION

Transcription of the IL-8 gene occurs rapidly when primary monocytes are stimulated with a variety of cytokines, such as IL-1, TNF, or IFNγ; however, at present it is unclear how the signals generated by these molecules result in enhanced gene expression. Cloning of IL-8 genomic sequences has identified possible binding sites for transcription factors such as AP-1, AP-2, HNF-1, IRF-1 and a glucocorticoid-responsive element [7] in addition to a potential NFκB binding site which may regulate IL-8 mRNA production [8]. An NFκB-like factor mediates the induction of the *gro* gene by IL-1 or TNF in fibroblasts [15], however any direct role of these factors in cytokine-mediated IL-8 gene expression remains to be demonstrated.

THP-1 and U937 cells have been used to investigate a number of monocyte signalling pathways, for example it has been shown that crosslinking of Fc receptors induces increases in intracellular calcium [13] and tyrosine phosphorylation [12,14]. The results reported here demonstrated that a combination of LPS and PMA gave optimal IL-8 gene expression, whereas PMA or LPS alone induced much lower expression. The responses of the cell lines appear to differ from those of primary monocytes, since LPS alone will induce IL-8 mRNA in primary cells [4]. IFNγ, IL-1, PAF, or fMLP did not induce IL-8 mRNA transcription in THP-1 or U937 cells. Adherence of monocytes to plastic induces novel gene expression [16], therefore it was possible that the mechanism of PMA-induced IL-8 gene expression was due to PMA-induced adherence of the monocytic cells to the tissue culture substratum. We found similar levels of IL-8 mRNA in adherent and nonadherent PMA- or PMA/LPS-treated cells, thus IL-8 gene expression is induced directly by PMA and is not an indi-

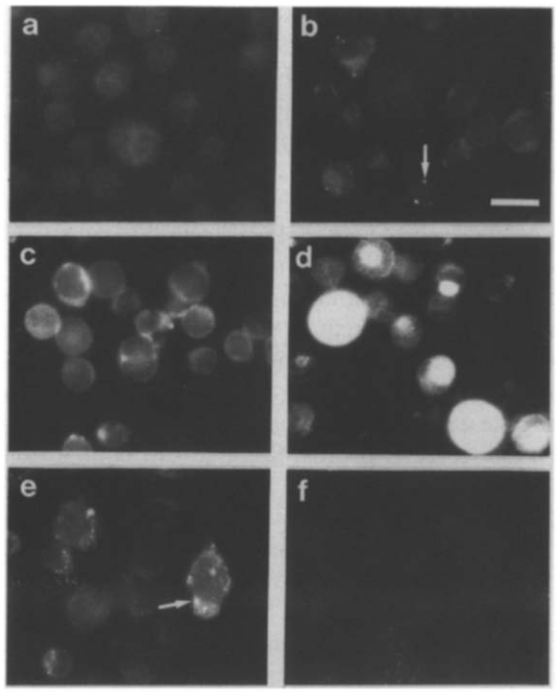

Fig. 3. Immunofluorescent localisation of IL-8 in THP-1 cells. Cells were treated as described for Fig. 1, fixed and stained for IL-8. (a) Untreated control cells. (b) LPS-stimulated cells. (c) PMA-stimulated cells. (d) PMA plus LPS-stimulated cells. (e) A23187-stimulated cells. (f) Untreated cells stained with pre-immune serum. Bar = 10 μm.

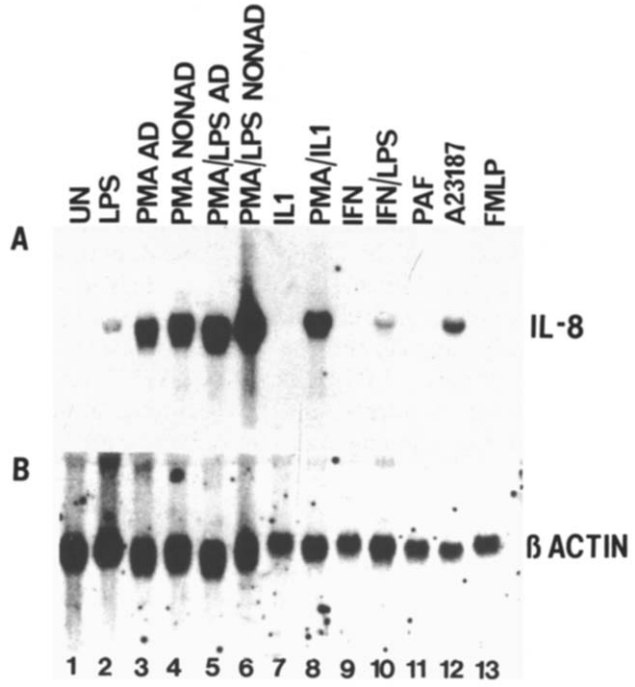

Fig. 2. (A) Expression of IL-8 mRNA in U937 cells in response to agonists or cytokine combinations. Cells were treated for 18 h with: 1 μg/ml LPS (lane 2); 10 ng/ml IL-1β (lane 7); 10 ng/ml PMA plus 10 ng/ml IL-1β (lane 8); 1000 U/ml IFNγ (lane 9); IFNγ plus 1 μg/ml LPS (lane 10); 1 μM platelet activating factor (lane 11); 1 μM A23187 (lane 12); 10^{-7} M fMLP (lane 13). In lanes 3–6 the cells were divided into PMA-treated adherent (lane 3) and nonadherent (lane 4) cells or PMA plus LPS-treated adherent (lane 5) and nonadherent (lane 6) cells. Lane 1 shows untreated cells. (B) The same blot re-probed for β-actin as loading controls.

Table I

IL-8 secretion by THP-1 cells

Treatment	IL-8 released into supernatant (ng/ml/10^6 cells ± S.E.M., n = 3)
None	0.4 ± 0.1
PMA (10 ng/ml)	11.0 ± 0.6
LPS (1 μg/ml)	2.4 ± 1.1
PMA → LPS	79.3 ± 11.2
A23187 (1 μM)	10.0 ± 1.1

Cells were treated as described for Fig. 1 and the amount of IL-8 released into the culture medium was quantified by ELISA. The amount released is expressed as ng/ml/10^6 cells and is the mean of 3 experiments.

rect consequence of PMA-induced cell adherence. A major novel finding reported here is that the calcium ionophore A23187 induced significant IL-8 mRNA levels in monocytic cell lines. This suggests that an increase in intracellular calcium is one mechanism by which the IL-8 gene can be activated. A number of other genes can be regulated by calcium, for example ionomycin induces immediate early genes in PC12 cells [9], and A23187 regulates transcription of the CD7 gene in T cells [10]. Calcium and calmodulin antagonists, whilst having no effect on their own, reduce the LPS-induced expression of the IP-10 and IL-1β genes in murine macrophages [11]. Recently it has been shown that A23187 induces IL-8 gene expression in human blood eosinophils and the levels of IL-8 protein secreted are comparable to those reported here [18]. Ionomycin augments IL-8 mRNA levels in response to PMA in T lymphocytes, but has no stimulatory effect on its own, indicating a role for intracellular calcium in regulating IL-8 gene expression in these cells [17]. In THP-1 and U937 cells A23187 alone was sufficient to induce IL-8 mRNA, protein synthesis and secretion, thus increases in intracellular calcium can lead to the production of a chemotactic factor from monocytic cells. It will be important to elucidate the other intracellular signals which will optimise IL-8 gene expression.

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