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Influence of interleukin 1α on superoxide anion, platelet activating factor release and phospholipase A_2 activity of naive and sensitized guinea-pig alveolar macrophages

[1]Sabrina Mugnai, Mario Ciuffi, Manuela Maurizi, Daniela Bindi, Sergio Franchi-Micheli & Lucilla Zilletti

Department of Preclinical and Clinical Pharmacology 'M. Aiazzi-Mancini', V.le G.B. Morgagni, 65; 50134 Florence, Italy

1 We studied the effect exerted by *hr*-interleukin-1α (IL-1α) on responsiveness of alveolar macrophages (AM) from naive and sensitized guinea-pigs, through $O_2^{\cdot-}$ production (by ferricytochrome C reduction), platelet-activating factor (PAF) release (by platelet aggregation), prostaglandin E_2 (PGE_2) release (by a radioimmunoassay), and cytosolic phospholipase A_2 ($cPLA_2$) activity (by hydrolysis of radioactive substrate).

2 In naive guinea-pig AM, 0.06 nM *hr*-IL-1α pretreatment decreased by 65% $O_2^{\cdot-}$ release stimulated with 10 nM fMLP. In contrast, $O_2^{\cdot-}$ production was not affected in sensitized guinea-pig AM.

3 $O_2^{\cdot-}$ release elicited by fMLP stimulation in both cell groups was affected by PLA_2 inhibitors (10 μM bromophenacyl bromide, BPB or 10 μM methylprednisolone, MP). In contrast, 10 μM arachidonyl trifluoromethyl ketone ($AACOCF_3$), a $cPLA_2$ inhibitor, was ineffective.

4 In naive AM, PAF release was elicited by *hr*-IL-1α pretreatment and by separate fMLP-stimulation, but when the stimulus was added to *hr*-IL-1α-pretreated cells inhibition of PAF release was observed. In sensitized AM, PAF release was lower than that found in naive guinea-pig AM in both *hr*-IL-1α-pretreated and fMLP-stimulated cells.

5 PGE_2 release was unaffected by *hr*-IL-1α pretreatment and it was decreased by fMLP in both naive and sensitized AMs. The latter released less PGE_2 than naive cells in basal conditions and after fMLP treatment.

6 Sensitized AM showed a greater $cPLA_2$ activity in all experimental conditions in comparison to naive cells. $cPLA_2$ activity assayed in the cytosolic fraction was found to be enhanced by *hr*-IL-1α pretreatment and by fMLP stimulation in naive but not in sensitized AM. However, when the stimulus was added to *hr*-IL-1α-pretreated cells we observed a decrease in $cPLA_2$ activity in the cytosol and an increase in the membranes, thus suggesting a translocation of enzymatic activity.

7 In conclusion, *hr*-IL-1α can modulate the responsiveness of AM from naive and sensitized guinea-pigs, as suggested by changes found in the release of PAF and $O_2^{\cdot-}$ and in $cPLA_2$ activity; therefore, sensitization itself may affect cellular responsiveness.

Keywords: Alveolar macrophages; sensitization; cytosolic phospholipase A_2 activity; platelet-activating factor; superoxide anion; cytokines

Introduction

There is considerable evidence that macrophages play an important role in asthma (Fuller, 1992). It is believed that the loss of suppressive activity of macrophages versus T-cell mediated immune-system may lead to excessive T-cell activation and chronic inflammation in this disease (Poulter *et al*., 1994).

Moreover, macrophages may modify their behaviour in response to various stimuli from either the external environment or other cells. In fact, they possess a number of receptors for cytokines and mediators suitable for transmitting different signals the integration of which can modify the production and secretion of cytokines, mediators and other products such as radical species of oxygen (Adams & Hamilton, 1992).

In particular, alveolar macrophages (AM) are known to be the major producers of proinflammatory cytokine interleukin-1 (IL-1) and to also possess receptors for this cytokine (Dinarello, 1991).

IL-1 causes a large spectrum of cellular responses, many of which are implicated in the pathology of asthma. Among these, the stimulation of arachidonic acid metabolism and platelet-activating factor (PAF) production appear to have considerable importance in this disease (Dinarello, 1991; Bussolino *et al*., 1994). PAF and the products of cyclo-oxygenase and lipoxygenase activity are known for their role as bronchoconstrictors and/or cellular chemoattractants and activators (Rankin, 1989).

It is generally accepted that among the enzymes determining the rate-limiting step in eicosanoid and PAF formation, phospholipases A_2 (PLA_2s EC 3.1.1.4. phosphatide sn-2 acylhydrolase) play a role (Glaser *et al*., 1993). PLA_2s, enzymes which are able to hydrolyze the sn-2-ester bond in phospholipids, exist in distinct forms with different molecular mass, substrate specificity, structure and catalytic mechanism (Mukherjee *et al*., 1994). Among these, the 85 kDa cytosolic PLA_2 ($cPLA_2$) has been shown to prefer the arachidonic acid in sn-2-position, thus suggesting that this enzyme is responsible for arachidonic acid release and cascade. Moreover, the same enzyme appears to be dynamic in its function since it translocates to the membrane after cell activation in the presence of Ca^{2+} (Dennis *et al*., 1995).

Among the numerous factors regulating $cPLA_2$, there is proinflammatory cytokine IL-1α (as well as other cytokines), which is able to induce the synthesis and activation of this enzyme (Lin *et al*., 1992; Kramer & Sharp, 1995).

A useful animal model to study airway hypersensitivity and hyperresponsiveness is the sensitized guinea-pig (Smith, 1989).

[1] Author for correspondence.

Biochemical and functional differences between AM from naive and sensitized guinea-pigs have been observed. Under basal conditions, production of free arachidonic acid from AM of the sensitized guinea-pig was lower than that from AM of the naive guinea-pig, suggesting that sensitization affects arachidonic acid release from the lipid pool, modifying the activity of enzymes involved in the deacylation and/or reacylation process (M'Rini *et al*., 1994). In another study, AM from sensitized guinea-pigs produced an enhanced amount of thromboxane B_2 and a lower amount of prostaglandin E_2 (PGE_2) in comparison to naive cells in basal conditions, but not after stimulation (Brunelleschi *et al*., 1992).

The aim of this study was to clarify the influence exerted by *hr*-IL-1α on AM responsiveness from naive and sensitized guinea-pigs, in both basal conditions or following stimulation with formyl-methionyl leucyl phenylalanine (fMLP). We performed this study by assay of $O_2^{\cdot}$ as a biochemical parameter of cell activation and by evaluation of PAF and PGE_2 release and $cPLA_2$ activity as measures of lipid metabolism.

Methods

Sensitization of guinea-pigs

Male guinea-pigs (300–400 g) were actively sensitized by s.c. injection (0.5 ml) of ovalbumin (0.5 mg kg^{-1}) plus incomplete Freund's adjuvant and used 4–6 weeks later (Dale, 1965).

Isolation of alveolar macrophages

Cells were harvested according to Myrvik *et al*. (1961). Tracheae of anaesthetized guinea-pigs (Pentothal 80 mg kg^{-1}, i.p.) were incubated and 60 ml of PBS were injected in aliquots of 5 ml. The fluid was collected and centrifuged (700 *g* for 10 min). After hypotonic lysis of erythrocytes the cells were plated (10^6 cells/well for $O_2^{\cdot}$ and PGE_2 assay or 4×10^6 cells/well for PAF and $cPLA_2$ activity assay) for 2 h (5% CO_2, 37°C), in RPMI 1640 with 10% FBS, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (complete RPMI). Then the cells were washed three times with HBSS to remove the non-adherent cells. Viability, evaluted by Trypan Blue exclusion, exceeded 98%.

Cells obtained from naive guinea-pigs are referred to as naive AM, and those obtained from sensitized guinea-pigs as sensitized AM.

hr-*IL-1α-pretreatment of AM*

Cells from naive and sensitized guinea-pigs were incubated for 18 h in complete RPMI with *hr*IL-1α or medium alone.

After incubation the cells were washed three times with HBSS and used for the activation with fMLP.

Treatment of AM with PLA_2 inhibitors

PLA_2 inhibitors were introduced to evaluate the influence on $O_2^{\cdot}$ production.

In some experiments, the adherent cells were pretreated for 1 h with 10 μM 4-bromophenacyl bromide (BPB) (Smolen & Weissmann, 1980; Duke *et al*., 1986) then washed three times with HBSS and afterwards incubated with *hr*-IL-1α for 18 h.

In other samples, 10 μM methylprednisolone (MP) was added to the adherent cells, simultaneously with the *hr*-IL-1α, during the incubation.

In another set of experiments the adherent cells were incubated with medium alone for 18 h, then washed three times with HBSS and treated for 2 min with or without $AACOCF_3$ (1–100 μM). In all experiments tests were performed in the absence of inhibitors and/or of *hr*-IL-1α-treatment.

In experiments carried out to check the inhibitory effect of $AACOCF_3$ on $cPLA_2$ activity, the concentration used (10 μM) was that shown to induce maximal inhibition in platelets (Riendeau *et al*., 1994).

Activation of AM

The AM were activated by incubation in 10 nM fMLP (Brunelleschi *et al*., 1992). The incubation time was 60 min when $O_2^{\cdot}$ and PGE_2 production were being measured and 5 min when $cPLA_2$ activity or PAF release (the last in presence of 0.25% BSA) were evaluated. The 60 min incubation time was selected since it was the time for maximal production of $O_2^{\cdot}$ in our conditions. Experiments in the absence of fMLP were always performed.

Superoxide anion assay

$O_2^{\cdot}$ production was evaluated by superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C (80 μM) (Brunelleschi *et al*., 1992). fMLP and ferricytochrome C were always added simultaneously. The changes in absorbance were detected at 550 nm by use of an extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} (Massey, 1959).

PAF release assay

PAF release was bioassayed by rabbit platelet aggregation according to Fitzgerald *et al*. (1989). PAF was extracted from the supernatant by chloroform in the presence of acetone. After evaporation to dryness under N_2, the residue was dissolved in Tris plus BSA 0.25%, pH 8, and bioassayed in washed platelets according to Eliakim *et al*. (1988).

In order to check if PAF release was measured correctly, we added exogenous PAF to the cells. When PAF (0.1, 0.3 and 3 ng ml^{-1}) was added to naive and sensitized AM, we recovered 0.073 ± 0.006, 0.225 ± 0.016 and 1.47 ± 0.098 ng ml^{-1} PAF from naive AM, and 0.067 ± 0.04, 0.235 ± 0.018 and 1.53 ± 0.130 ng ml^{-1} PAF from sensitized AM. Therefore, in the PAF concentration range released by cells (from 0.1 to 1.1 ng ml^{-1}), PAF recovered was about 75% of exogenous PAF standard added.

PGE_2 assay

The PGE_2 release was assayed according to Ciuffi *et al*. (1996). Cellular supernatant was extracted with 2 ml ethylacetate and centrifuged (1000 *g*, for 10 min, 0°C); 1.5 ml of the organic phase was placed in polypropylene tubes and evaporated to dryness at room temperature by an Argon stream. The dry residue was dissolved in buffer (0.0025 M NaH_2PO_4, 0.01 M EDTA, 0.3% bovine globulin in 0.9% NaCl), pH 6.8, and assayed according to the manufacturer's instructions after suitable dilutions. Aliquots (in triplicate of 100 μl) were used. The radioactive label was ^{125}I ([^{125}I]-PGE_2 specific activity 2200 Ci $mmol^{-1}$) and PGE_2 antiserum showed a cross reactivity of $<2.5\%$ with other prostaglandins.

$cPLA_2$ activity assay

Enzymatic activity were assayed according to Wu *et al*. (1994). Briefly, the cells were scraped into 500 μl of homogenate buffer (50 mM HEPES pH 8, 0.1 mM EDTA, 1 mM EGTA, 50 μg ml^{-1} leupeptin, 1 mM dithiothreitol, 10 μg ml^{-1} soybean trypsin inhibitor, 0.5 μM phenylmethylsulphonyl fluoride, 10 μM phosphoramidon, 100 μg ml^{-1} aprotinin) and sonicated for 4×15s. After centrifugation (1000 *g* for 5 min), the supernatant was ultracentrifuged (100,000 *g* for 1 h) and a soluble fraction, called the crude cytosol, was obtained. The remaining particulate fraction was separated and washed twice in 150 μl homogenate buffer and resuspended in this buffer plus 0.05% Triton X-

100. This suspension was sonicated for 5 s and incubated for 30 min on ice: this fraction was called the crude membrane.

The reaction mixture included: 15 μM L-α-1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine, 2.5 μl 116 mM $CaCl_2$, 95.5 μl of crude cytosol or membrane fractions; it was incubated for 1 h at 37°C. Then 300 μl of 2:1 chloroform:methanol containing 1% acetic acid and 3 mM arachidonic acid, was added.

The organic phase was analysed by thin layer chromatography (t.l.c., heptane:isopropyl ether:acetic acid 60:40:4 was used as eluent). The spot with the same R_F of standard arachidonic acid was scraped. Released arachidonic acid was quantified by scintillation counting and expressed as pmol arachidonic acid hydrolyzed mg^{-1} protein h^{-1}.

Protein assay

Protein was assayed according to Lowry *et al*. (1951).

Statistical analysis

Data are presented as means ± s.e.mean. The statistical differences were evaluated by Student's *t* test ('grouped data' or 'paired data' as appropriate). A probability value of $P<0.05$ was regarded as significant.

Drugs

Animals were obtained from Morini (Reggio Emilia, Italy); tissue culture reagents (phosphate-buffered saline (PBS), RPMI 1640, Hank's balanced salt solution (HBSS), penicillin-streptomycin, foetal bovine serum heat inactivated (FBS) were obtained from GIBCO BRL (Paisley, U.K.); *hr*-IL-1α from Peprotech Inc. (Rocky Hill, NJ, U.S.A.); BSA from Boheringer (Mannheim, Germany); t.l.c. plates silica gel 60 from Merck (Darmstadt, Germany); L-α-1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine, 57 mCi $mmol^{-1}$, from Du Pont NEN (FGR); PGE_2 radioimmunoassay NEK020 kit from New England, Nuclear (NEN Chemicals GmbH Dreiech, Germany); incomplete Freund's adjuvant from Difco (Detroit, MI, U.S.A.); pentothal from Abbott (Latina, Italy); arachidonyl trifluoromethyl ketone ($AACOCF_3$) from Biomol (Plymouth Meeting, PA, U.S.A.); methylprednisolone, 4-bromophenacyl bromide, β-acetyl-γ-O-alkyl-L-α-phosphatidylcholine or AcGEPC (PAF) and superoxide dismutase from Sigma (St. Louis, Mo, U.S.A.). All the reagents were of the highest analytical grade commercially available.

Results

Effect of hr-*IL-1α pretreatment on* $O_2^{\cdot-}$ *release*

Basal $O_2^{\cdot-}$ production in naive or in sensitized AM was not affected by pretreatment with 0.06 nM *hr*-IL-1α (Figure 1). Similar behaviour was observed in both cell groups when a higher dose (0.6 nM) of cytokine was used (0.98 ± 0.17 and 0.83 ± 0.25 nmol $O_2^{\cdot-}/10^6$ cells in naive and sensitized AM, respectively, $n=8$).

Pretreatment with 0.06 nM *hr*-IL-1α clearly decreased $O_2^{\cdot-}$ release (about 65%) in response to 10 nM fMLP in naive AM (Figure 1). Moreover, after pretreatment with a higher concentration (0.6 nM) of *hr*-IL-1α followed by stimulation with fMLP, a significant ($P<0.025$) inhibitory effect on superoxide anion production was observed (3.76 ± 0.24 nmol $O_2^{\cdot-}/10^6$, $n=8$). In sensitized AM, fMLP-stimulated $O_2^{\cdot-}$ production was not significantly different from the value of naive cells but, in contrast, it was not affected by 0.06 nM *hr*-IL-1α pretreatment (Figure 1). A higher dose (0.6 nM) of *hr*-IL-1α was also unable to modify fMLP-stimulated $O_2^{\cdot-}$ production (10.63 ± 0.94 nmol $O_2^{\cdot-}/10^6$ cells, $n=8$).

Effect of PLA_2 *inhibitors on* $O_2^{\cdot-}$ *release*

When the cells were pretreated for 1 h with 10 μM BPB, a nonspecific and irreversible PLA_2 inhibitor, we observed that basal $O_2^{\cdot-}$ production was not affected. When *hr*-IL-1α incubation was performed, BPB treatment did not modify the $O_2^{\cdot-}$ release in either naive or sensitized cells (Table 1). In contrast, we observed inhibition of fMLP-stimulated $O_2^{\cdot-}$ release in both naive and sensitized AM (Table 1). This inhibition was partially reversed by the addition of 50 μM arachidonic acid 15 min before stimulation with fMLP; in this condition, 7.12 ± 1.13 nmol $O_2^{\cdot-}/10^6$ cells and 6.37 ± 1.43 nmol $O_2^{\cdot-}/10^6$ cells ($n=6$) were produced in naive and sensitized AM, respectively (BPB-treated and fMLP-stimulated). In the absence of fMLP stimulation arachidonic acid did not have a significant effect on $O_2^{\cdot-}$ production (1.22 ± 0.26 and 0.90 ± 0.11 nmol $O_2^{\cdot-}/10^6$ cells in naive and in sensitized AM, respectively, $n=6$).

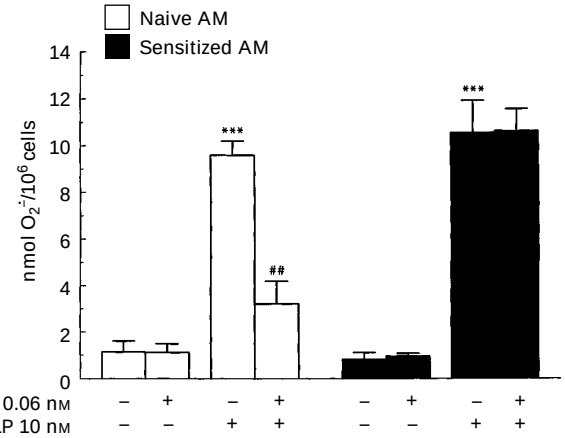

Figure 1 Superoxide anion production, unstimulated or fMLP-stimulated, from untreated or *hr*-IL-1α-pretreated AM. Cells were incubated for 18 h with or without 0.06 nM *hr*-IL-1α, then washed and incubated with saline or 10 nM fMLP for 60 min. Data are presented as means ± s.e.mean; $n=10$. The statistical differences were evaluated by Student's *t* test 'paired data'. $^{***}P<0.005$ vs basal; $^{\#\#}P<0.025$ vs fMLP.

Table 1 Effect of 10 μM BPB or 10 μM MP on $O_2^{\cdot-}$ production by untreated or 0.06 nM *hr*-IL-1α-pretreated AM, stimulated with 10 nM fMLP

	$O_2^{\cdot-}$ *production* (nmol/10^6 cells)	
	Naive	*Sensitized*
Basal	0.91 ± 0.11	0.98 ± 0.28
BPB	0.97 ± 0.30	0.77 ± 0.32
MP	1.16 ± 0.56	0.86 ± 0.15
hr-IL-1α	1.25 ± 0.39	0.90 ± 0.23
BPB + *hr*-IL-1α	1.28 ± 0.41	0.83 ± 0.19
MP + *hr*-IL-1α	1.35 ± 0.42	0.94 ± 0.07
fMLP	9.80 ± 0.51	10.41 ± 0.78
BPB + fMLP	1.93 ± 0.38***	2.36 ± 0.33***
MP + fMLP	3.12 ± 0.40**	4.26 ± 0.77**
hr-IL-1α + fMLP	3.45 ± 0.52	10.35 ± 1.09
BPB + *hr*-IL-1α + fMLP	2.62 ± 0.69	3.31 ± 0.94**
MP + *hr*-IL-1αf + MLP	2.85 ± 0.87	6.19 ± 0.43*

Cells were incubated for 1 h with or without 10 μM BPB, then washed and incubated for 18 h with or without 0.06 nM *hr*-IL-1α. Other cells, were incubated with 10 μM MP added simultaneously with *hr*-IL-1α and incubated for 18 h. Finally the cells were washed and incubated with saline or 10 nM fMLP for 1 h. Data are presented as mean ± s.e.mean; $n=8$. The statistical differences were evaluated by Student's *t* test 'paired data'. $^{*}P<0.05$; $^{**}P<0.025$; $^{***}P<0.005$ vs its control.

Table 1 also shows the effect of 10 μM BPB on $O_2^{\cdot-}$ release from *hr*-IL-1α-pretreated and fMLP-stimulated AM of naive and sensitized guinea-pigs. We observed that in these conditions, BPB did not modify $O_2^{\cdot-}$ release in naive cells, but it exerted a significant inhibitory effect in sensitized cells.

Table 1 also shows that $O_2^{\cdot-}$ release from naive and sensitized AM was not affected by treatment for 18 h with 0.06 nM *hr*-IL-1α and MP. But MP inhibited fMLP-stimulated $O_2^{\cdot-}$ production in both cell groups. When the cells incubated with MP and *hr*-IL-1α were afterwards stimulated with fMLP, we observed a decrease in $O_2^{\cdot-}$ production in sensitized AM in comparison to that found in the absence of MP. No significant variations were observed in naive cells (Table 1).

The 10 μM MP dose was used because previous experiments had shown that fMLP-evoked $O_2^{\cdot-}$ production of naive guinea-pig AM was reduced by MP in a dose-dependent manner, with a maximal reduction at 10 μM (Figure 2). No effects on unstimulated release were observed (1.15 ± 0.24; 1.32 ± 0.50; 1.07 ± 0.35 nmol $O_2^{\cdot-}/10^6$ cells produced in presence of 1 μM, 10 μM and 100 μM MP, respectively).

In naive and sensitized AM, treatment with the $cPLA_2$ inhibitor $AACOCF_3$ (1–100 μM) did not modify unstimulated or fMLP-stimulated $O_2^{\cdot-}$ release (Table 2).

Effect of hr-*IL-1α-pretreatment on PAF release*

The PAF release is shown in Figure 3. In naive AM both 0.06 nM *hr*-IL-1α-pretreatment and 10 nM fMLP-treatment caused PAF release; but we observed a reduction in PAF release when fMLP stimulation was performed in *hr*-IL-1α-pretreated AM; this value was lower than that found with fMLP or *hr*-IL-1α alone. In sensitized AM, fMLP was elicted PAF release, although to a smaller extent than in naive cells ($P<0.05$

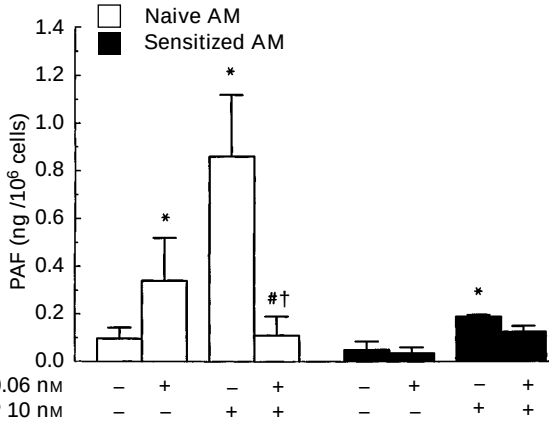

Figure 3 PAF release induced by untreated or *hr*-IL-1α-pretreated AM, in the absence or presence of 10 nM fMLP. Cells were incubated for 18 h with or without 0.06 nM *hr*-IL-1α, then washed and incubated with saline or 10 nM fMLP for 5 min. Data are presented as mean±s.e.mean; $n=6$. The statistical differences were evaluated by Student's *t* test 'paired data'. $^*P<0.05$ vs untreated cells; $^{\#}P<0.05$ vs *hr*-IL-1α; $\dagger P<0.025$ vs fMLP.

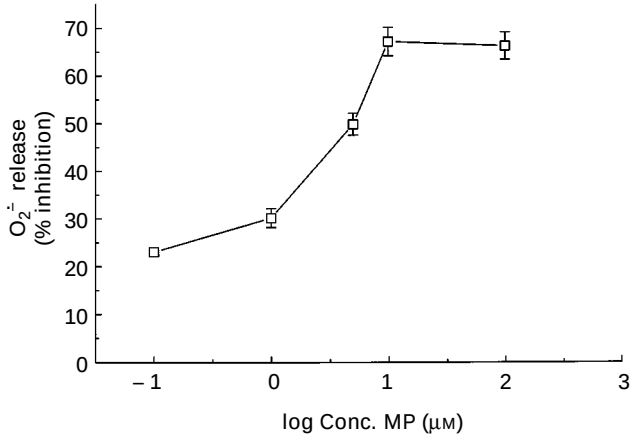

Figure 2 The dose-dependent inhibitory effect of MP on fMLP-stimulated superoxide release. Naive AM incubated for 18 h with MP at various concentrations, were stimulated with 10 nM fMLP for 1 h and the $O_2^{\cdot-}$ production was detected. The % inhibition was calculated versus the fMLP-stimulated $O_2^{\cdot-}$ production in absence of MP (9.59 ± 0.48 nmol $O_2^{\cdot-}/10^6$ cells).

Table 2 Effect of $AACOCF_3$ on $O_2^{\cdot-}$ production by naive AM, stimulated with 10 nM fMLP

	$O_2^{\cdot-}$ *production* (nmol/10^6 cells)	
	Naive	*Sensitized*
Basal	1.11 ± 0.34	0.88 ± 0.25
$AACOCF_3$ 10 μM	1.17 ± 0.36	1.32 ± 0.27
$AACOCF_3$ 100 μM	0.96 ± 0.32	1.05 ± 0.43
fMLP	10.46 ± 1.16	10.35 ± 1.45
$AACOCF_3$ 1 μM + fMLP	10.36 ± 1.47	10.73 ± 1.34
$AACOCF_3$ 10 μM + fMLP	10.69 ± 1.15	11.16 ± 1.94
$AACOCF_3$ 100 μM + fMLP	10.81 ± 1.55	10.49 ± 1.63

Naive and sensitized AM were treated for 2 min with $AACOCF_3$ (1–100 μM), and stimulated (or unstimulated) with 10 nM fMLP. The $O_2^{\cdot-}$ production was assayed as described in Methods. Data are mean±s.e.mean, $n=3$.

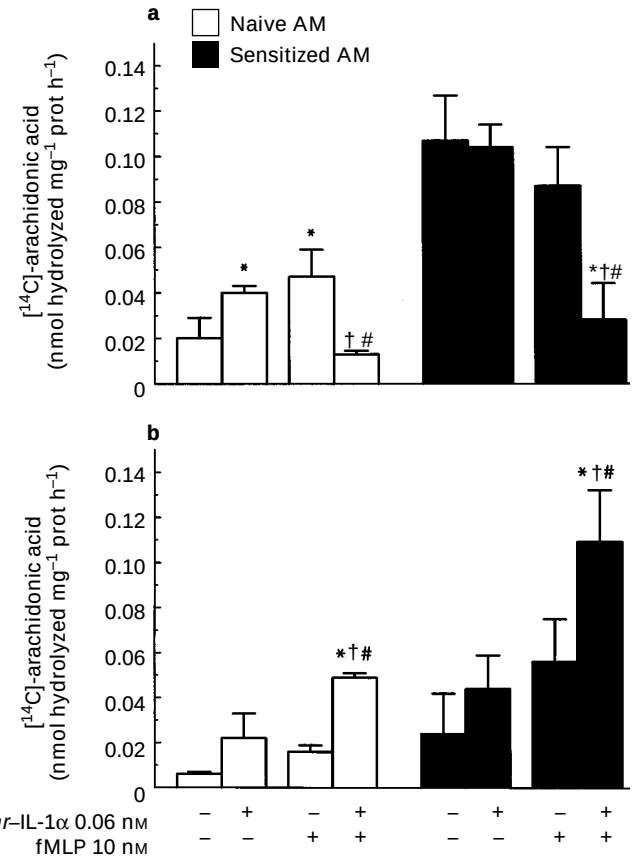

Figure 4 $cPLA_2$ activity in unpretreated or *hr*-IL-1α-pretreated AM, in the absence or presence of 10 nM fMLP. Cells were incubated with or without 0.06 nM *hr*-IL-1α for 18 h, then washed and incubated with saline or 10 nM fMLP for 5 min. The cells were scraped and the cytosol and membrane fractions were prepared as described in Methods. (a) $cPLA_2$ activity assayed in cytosol; (b) $cPLA_2$ assayed in membrane fraction. Data are presented as mean±s.e.mean; $n=5$. The statistical differences were evaluated by Student's *t* test 'paired data'. $^*P<0.05$ vs basal; $^{\#}P<0.05$ vs fMLP alone; $\dagger P<0.05$ vs *hr*-IL-1α alone.

as evaluated by Student's *t* test 'grouped data'); *hr*-IL-1α pretreatment did not modify either basal or fMLP-stimulated PAF release. The value found in *hr*-IL-1α-pretreated cells was less than that in naive cells ($P<0.05$ as evaluated by Student's *t* test 'grouped data') in the same conditions.

Effect of hr-*IL-1α pretreatment on cPLA$_2$ activity*

The activity of cPLA$_2$ assayed in the cytosolic fraction of AM is shown in Figure 4a. In naive AM, pretreatment with either 0.06 nM *hr*-IL-1α or with 10 nM fMLP enhanced cPLA$_2$ activity compared to the basal value. When the naive cells were pretreated with 0.06 nM *hr*-IL-1α and then stimulated with fMLP, we observed a decrease in cPLA$_2$ activity in comparison to *hr*-IL-1α or fMLP alone.

In sensitized AM, basal enzymatic activity was found to be increased in comparison with naive cells ($P<0.05$ as evaluated by Student's *t* test 'grouped data') and was not affected by *hr*-IL-1α pretreatment or by fMLP treatment. Figure 4a also shows an evident decrease of cPLA$_2$ activity in *hr*-IL-1α-pretreated and fMLP-stimulated sensitized cells, in comparison to enzymatic activity found in basal conditions or after *hr*-IL-1α pretreatment or fMLP stimulation alone.

In Figure 4b cPLA$_2$ activity assayed in the membrane fraction of naive and sensitized AM is shown. In both cases we observed that neither pretreatment with *hr*-IL-1α, nor treatment with fMLP affected the basal enzymatic activity. When the *hr*-IL-1α-pretreated cells were stimulated with fMLP, we observed an increase in cPLA$_2$ activity compared to basal fMLP and *hr*-IL-1α values in both naive and sensitized cells. The sum of the values of enzymatic activity found in cytosolic and membrane fractions for each experimental condition obtained are presented in Table 3. It was found that all the values obtained in sensitized AM were higher than those in naive AM.

Table 4 shows the effect of AACOCF$_3$ on cPLA$_2$ activity in fMLP-stimulated naive and sensitised AM. This inhibitor affected the basal and fMLP-stimulated enzymatic activity in both the cytosolic and membrane fractions.

Effect of hr-*IL-1α pretreatment on PGE$_2$ release*

PGE$_2$ production is shown in Figure 5. In naive AM *hr*-IL-1α pretreatment did not affect PGE$_2$ release, whereas we observed a decrease (about 50%) when the naive cells were stimulated with fMLP. *hr*-IL-1α pretreatment did not modify this effect of fMLP. In sensitized AM we observed the same trend of effects as in naive cells, but the values were significantly lower than those observed in naive AM, in basal conditions ($P<0.05$ as evaluated by Student's *t* test 'grouped data') and after *hr*-IL-1α pretreatment ($P<0.05$ as evaluated by Student's *t* test 'grouped data') or fMLP treatment ($P<0.01$ as evaluated by Student's *t* test 'grouped data').

Table 3 Total amount of cLPA$_2$ activity

	cPLA$_2$ activity (nmol [^{14}C]-arachidonic acid hydrolyzed mg^{-1} prot h^{-1})	
	Naive	*Sensitized*
Basal	0.029±0.016	0.156±0.017**
hr-IL-1α	0.060±0.0035$^{\#}$	0.148±0.0027**
fMLP	0.063±0.016$^{\#}$	0.112±0.024*
hr-IL-1α+fMLP	0.062±0.0018$^{\#}$	0.138±0.015**

The values were obtained by adding the amount of cytosolic enzymatic activity to that found in membranes. $^{\#}P<0.05$ vs basal (as evaluated by Student's *t* test 'paired data'). $^{*}P<0.05$; $^{**}P<0.02$ vs naive cells (as evaluated by Student's *t* test 'grouped data').

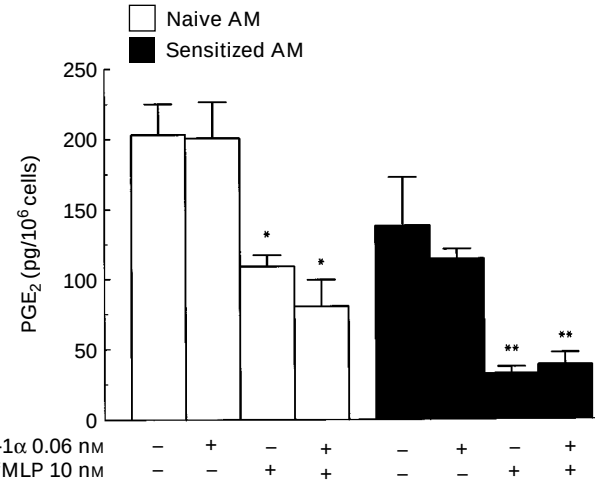

Figure 5 PGE$_2$ production in unstimulated or fMLP-stimulated, untreated or *hr*-IL-1α-pretreated AM. Cells were incubated with or without 0.06 nM *hr*-IL-1α, then washed and incubated with saline or 10 nM fMLP for 60 min. Data are presented as means±s.e.mean; $n=4$. The statistical differences were evaluated by Student's *t* test 'paired data'. $^{*}P<0.05$; $^{**}P<0.01$ vs basal.

Discussion

AM from naive or sensitized guinea-pigs showed different biochemical responses when they were preincubated with *hr*-IL-1α and/or stimulated with fMLP. We observed that in naive AM preincubated with *hr*-IL-1α cPLA$_2$ activity was enhanced in the cytosolic fraction and PAF was released, while in sensitized AM no variations in comparison to controls were apparent. The increment of cPLA$_2$ activity is an effect that IL-1 generally induces in other cells (Hulkower *et al*., 1992; Bomalaski *et al*., 1992; Lin *et al*., 1992; Gronich *et al*., 1994; see Dinarello, 1991 and Schütze *et al*., 1994 for reviews). In particular, this cytokine has been shown to be able to increase the synthesis of cPLA$_2$ (Schalkwijk *et al*., 1993; Angel *et al*., 1994); thus we cannot exclude the possibility that the enhanced cPLA$_2$ activity observed in our experiments may be related to an increased expression of the enzyme by *hr*-IL-1α.

The increase of PAF release that we have found in *hr*-IL-1α-pretreated naive AM was previously shown in other cell types, such as endothelial cells (Bussolino *et al*., 1994). The main enzymatic pathway leading to PAF synthesis involves the

Table 4 Effect of 10 μM AACOCF$_3$ on cPLA$_2$ activity in fMLP-stimulated naive and sensitized AM

	cPLA$_2$ activity (nmol [^{14}C]-arachidonic acid hydrolyzed mg^{-1} prot h^{-1})			
	Cytosol		*Membrane*	
	Naive	*Sensitized*	*Naive*	*Sensitized*
Basal	0.0176±0.0031	0.1047±0.027	0.0087±0.00014	0.0253±0.0016
fMLP	0.0433±0.0012	0.093±0.018	0.0118±0.0019	0.00403±0.0015
AACOCF$_3$	0.0083±0.0003*	0.0456±0.0038*	0.0015±0.00025*	0.0072±0.00024**
AACOCF$_3$+fMLP	0.0075±0.0002$^{\#\#}$	0.0423±0.0029$^{\#}$	0.0010±0.00009$^{\#\#}$	0.0077±0.00011$^{\#\#}$

Naive and sensitized AM were treated for 2 min with 10 μM AACOCF$_3$, then stimulated (or unstimulated) for 5 min with 10 nM fMLP. The enzymatic activity was assayed as described in Methods. Data are means±s.e.mean, $n=3$. $^{*}P<0.05$; $^{**}P<0.01$ vs Basal. $^{\#}P<0.05$; $^{\#\#}P<0.01$ vs fMLP.

production of lyso PAF by PLA_2s and then acetylation to PAF by acetyltransferase (Zimmerman *et al*., 1992; Bazan, 1995). In particular, it has been suggested that $cPLA_2$ may play an important role in PAF formation (Kramer & Sharp 1995). Our data are not in disagreement with this hypothesis, since we observed that PAF release exhibited a similar trend to that of $cPLA_2$ activity. However, it should be noted that other PLA_2s (such as secretory PLA_2, $sPLA_2$) may be involved in PAF synthesis. In naive AM, fMLP was able to increase PAF release and $cPLA_2$ activity in the cytosolic fraction. In this regard, PLA_2 activation following fMLP stimulation was previously hypothesized by Kadiri *et al*. (1990). When *hr*-IL-1α-pretreated naive AM were stimulated with fMLP we observed a reduction in PAF release. We can suppose that *hr*-IL-1α pretreatment may affect the fMLP-activated enzymatic pathway leading to PAF release. The increase in $cPLA_2$ activity that we observed in the membrane fraction could lead to higher intracellular levels of arachidonic acid which are able to inhibit acetyltransferase (Snyder, 1995).

Alternatively, it is possible that PLA_2s other than $cPLA_2$ (i.e.: $sPLA_2$) may be important in the first step of PAF synthesis. With regard to the $cPLA_2$ activity assayed in naive *hr*-IL-1α-pretreated AM, after stimulation with fMLP, we found a decrease in the cytosolic fraction but an increase in the membrane fraction, thus suggesting translocation of this enzyme from the cytosol to the membranes. This phenomenon has previously been observed in other cell types in the presence of different stimuli (Clark *et al*., 1991; Durstin *et al*., 1994).

When we assayed PGE_2 release, we found that *hr*-IL-1α pretreatment was unable to affect either basal or fMLP-evoked release. Previous studies have shown that IL-1 is able to induce PGE_2 release (Dinarello, 1991; Schütze *et al*., 1994), although this occurred in experimental conditions completely different from ours. On the contrary, we found a decrease (about 50% in eicosanoid release after fMLP-treatment. On the other hand, $cPLA_2$ activity was increased after *hr*-IL-1α pretreatment and after fMLP-stimulation suggesting that, in these conditions, $cPLA_2$ activity is not a rate limiting step in PGE_2 production. Other hypotheses are that free radical oxygen species are involved both indirectly, in the modulation of cyclo-oxygenase activity (Lands *et al*., 1984), and directly, in the synthesis of prostaglandins (Ciuffi *et al*., 1996).

The possibility that arachidonic acid (a product of PLA_2 activity) plays an important role in up-regulating $O_2^{\cdot-}$ production has been suggested previously (Bromberg & Pick, 1983; Sakata *et al*., 1987). In naive cells, stimulation with fMLP was able to produce $O_2^{\cdot-}$ and to increase $cPLA_2$ activity, in agreement with the above mentioned hypothesis. Thus, the enhanced $cPLA_2$ activity may lead to an increase in arachidonic acid concentration and subsequent stimulation of an oxidase producing $O_2^{\cdot-}$. However, this hypothesis seems to be invalidated if the effects of the PLA_2 inhibitors are considered. In our model, $AACOCF_3$ inhibited $cPLA_2$ activity in both cytosolic and membrane fractions, moreover, it is known that this arachidonic acid analogue at micromolar concentrations, also used by us, inhibits $cPLA_2$ but leaves $sPLA_2$ unaffected (Street *et al*., 1993). The presence of this molecule did not affect fMLP-stimulated $O_2^{\cdot-}$ release. On the other hand, BPB, acting mainly on $sPLA_2$ (Mayer & Marshall, 1993), showed a great ability to reduce the fMLP-stimulated $O_2^{\cdot-}$ production, while arachidonic acid was able to reverse this inhibition, as previously found (Henderson *et al*., 1989). A severe reduction in fMLP-stimulated $O_2^{\cdot-}$ release was also exhibited by MP, a $sPLA_2$ inhibitor (Glaser *et al*., 1993). In conclusion, we suggest that the last enzyme ($sPLA_2$) is involved in oxygen free radical production. Moreover, we point out that in our experiments the cells, after 18 h of incubation, were repeatedly washed before stimulation with fMLP, so that only the $sPLA_2$ still cell associated (Hidi *et al*., 1993) seems to be implicated in this process. However, as the pretreatment with $AACOCF_3$ left the production of $O_2^{\cdot-}$ unaffected, the role of $cPLA_2$ remains to be clarified.

There is controversal evidence concerning the effect of IL-1 on $O_2^{\cdot-}$ production. In some studies, IL-1 stimulated oxidative burst (Sample & Czuprynski, 1991; Tao *et al*., 1993), but not in others (Georgilis *et al*., 1987; Dularay *et al*., 1990). In our experimental conditions, pretreatment with *hr*-IL-1α was unable to elicit $O_2^{\cdot-}$ production in either naive or sensitized AM. On the other hand, when naive *hr*-IL-1α-pretreated cells were stimulated with fMLP, we observed a decrease in $O_2^{\cdot-}$ production. This suggests that *hr*-IL-1α pretreatment may interfere with fMLP-stimulated pathways leading to $O_2^{\cdot-}$ production. Alternatively, since in some cell types IL-1 was able to induce SOD (Harris *et al*., 1991), a similar mechanism could have occurred in naive cells, in our experiments.

hr-IL-1α-pretreatment or fMLP-stimulation in sensitized AM was unable to increase $sPLA_2$ activity in the cytosol or in the membrane. On the contrary when the stimulus was added to *hr*-IL-1α-pretreated cells, we observed significant changes in both fractions, with translocation of the activity from the cytosol to the membrane. The last effect was also shown in naive cells.

We emphasize that in the cytosolic fraction of sensitized AM, $cPLA_2$ activity either in basal conditions or following *hr*-IL-1α pretreatment was higher than that present in naive cells. Moreover, in all experimental conditions the total amount of PLA_2 activity (cytosolic + membrane) was significantly higher than that of unsensitized cells. We suggest that in sensitized AM the enzyme is in greater amounts than in naive cells or alternatively that mechanisms regulating PLA_2 activation are modified in sensitized cells. Therefore, this observation may indicate that after sensitization, deep changes in biochemical machinery implicated in cell responsiveness occur, as previously hypothesized (Beusemberg *et al*., 1991). Sane *et al*. (1996) found that sensitized guinea-pig cells from bronchoalveolar lavage showed an increase in $sPLA_2$ activity but not in $cPLA_2$. But Western blot of $cPLA_2$ showed controversal results in control cells and the amount of protein did not correlate with enzymatic activity.

It is very difficult to explain the meaning of the results concerning PAF and PGE_2 release, which after *hr*-IL-1α pretreatment or fMLP stimulation of sensitized AM, were significantly lower than those found in naive cells. A decrease in biosynthesis rate or a turnover increase for the mediators could be involved. Moreover, we observed that PAF and PGE_2 release seem to be unaffected by *hr*-IL-1α pretreatment in both unstimulated or fMLP-stimulated sensitized AM. This may suggest, in sensitized cells, a lesser responsiveness to *hr*-IL-1α rather than in naive cells. Concerning fMLP stimulation, sensitized AM showed the same behaviour as naive cells (i.e. PAF increase and PGE_2 decrease), although at values lower than those found in naive cells.

In sensitized AM, *hr*-IL-1α pretreatment does not stimulate $O_2^{\cdot-}$ release as previously observed in naive cells. Moreover, this pretreatment was not able to modify $O_2^{\cdot-}$ release in response to fMLP. When the stimulus was added to either unpretreated or *hr*-IL-1α-pretreated cells, $O_2^{\cdot-}$ release was sensitive to the action of $sPLA_2$ inhibitors tested, but not to $AACOCF_3$. These data suggest that the $sPLA_2$ may be important in stimulating respiratory burst. Moreover, mechanisms regulating $O_2^{\cdot-}$ production may be less sensitive to *hr*-IL-1α pretreatment in sensitized than in naive cells.

In conclusion, *hr*-IL-1α pretreatment can modulate cell responsiveness in different ways in naive and sensitized AM, as shown by the changes found in PAF and $O_2^{\cdot-}$ release and in $cPLA_2$ activity. The role of this enzyme in PAF and PGE_2 production in our model remains to be clarified.

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