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Phosphoinositide breakdown and superoxide anion release in formyl-peptide-stimulated human alveolar macrophages

Comparison between quiescent and activated cells

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Human alveolar macrophages (AMs) from allergic asthmatics (AAs) showed continuous Li^+-sensitive production of IP_1, indicating that the cells were continuously activated. Furthermore, whereas the accumulation of IP_1, IP_2 and IP_3 rapidly increased by as much as 125–175% in chemotactic-factor-stimulated AMs from healthy subjects, stimulation of cells from AAs increased these inositol phosphates only slightly. This moderate production could be due to a permanent state of activation leading to a depleted pool of polyphosphoinositides, corroborating the greater capacity of these cells to generate superoxide anion after stimulation by a chemoattractant. The activation state could be due to the action of priming agents, which are known to be released into the inflammatory sites.

Phosphoinositide; Superoxide anion; Formyl peptide; Asthma; (Alveolar macrophage)

1. INTRODUCTION

Phagocytes can be activated by soluble stimuli, such as chemotactic factors, to increase cellular enzyme release, motility, oxygen consumption, and the production of oxygen species [1–4]. Among these cells, human alveolar macrophages (AMs) play an important role in the local inflammatory process associated with bronchial hyperreactivity in asthma [5]. After specific stimulation, AMs from asthmatics are capable of a respiratory burst leading to the production of reactive oxygen species [6,7] known to promote tissue damage [8,9]. Formyl peptides, which are considered to be important chemoattractants involved in cell recruitment to local inflammatory sites [10], promote the release of superoxide anion ($O_2^{\cdot -}$). Specific receptors for these substances have been identified on the AM membrane [11,12]. The first step in formyl-peptide activation is its reversible binding to specific membrane-surface receptors [13,14]. A number of intracellular events can follow stimulus recognition [16–19], such as an increase in polyphosphoinositide (PPI) turnover [15]. A phospholipase C is activated which cleaves the inositol phosphate portion from polyphosphoinositides yielding the two second messengers, diacylglycerol and inositol phosphates [20,21].

There is no work in the literature on phosphoinositide breakdown in AMs related to an activated-cell state in asthma. Thus, our aim was to compare phosphatidylinositol turnover and the production of $O_2^{\cdot -}$ in quiescent and activated AM stimulated in vitro by *N*-formyl-leucyl-phenylalanine.

2. MATERIALS AND METHODS

2.1. *Human AM harvest and culture*

Alveolar cells were obtained by bronchoalveolar lavages per-

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formed in twelve allergic asthmatic patients (AA) and thirteen healthy subjects (HS) with a mean age of 40 ± 6 years. Bronchial asthma was diagnosed according to criteria of the American Thoracic Society [22]. BAL fluids were collected by gentle aspiration of 0.9% saline instillated as previously described [23]. Cells were harvested by centrifugation and viability was evaluated by the trypan-blue exclusion test. AMs were then purified by adherence to plastic Petri dishes (35 × 10 mm, Becton Dickinson, Grenoble, France) in 2 ml of medium 199 containing heat-inactivated fetal calf serum (20%), penicillin (50 U/ml) and streptomycin (50 μg/ml) (Gibco Chemical Co., Glasgow, Scotland). AMs were incubated in a humid atmosphere of 95% air and 5% CO_2 for 2 h at 37°C before experiments. The percentage of AMs was evaluated in duplicate culture plates by studying latex particle phagocytosis. Their viability was assessed by determining lactate dehydrogenase activity [24]. Each assay was performed with 0.5–1 × 10^6 AMs.

2.2. *Assay for superoxide anion generation*

Superoxide anion release was measured by superoxide dismutase inhibitable reduction of ferricytochrome *c* according to Johnston et al. [25]. Briefly, plated cells were incubated in 1 ml of a solution of 80 μM ferricytochrome *c* in Hank's balanced salt solution (cytochrome type IV from Sigma, St. Louis, MO). FMLP (10^{-7} M, Sigma) was added to the incubation mixture 1, 5 and 15 min before the assay. At the end of incubation, absorbance was determined (absorbance at 550 nm measured with a PU 8720 UV/VIS scanning spectrophotometer, Philips, France) using mixtures from plates without cells as blanks. Each reaction was run in duplicate. O_2^- release is expressed as nmol/10^6 AMs, and results as means ± SE. Statistical significance in AMs from AA compared to those from HS was evaluated by the Mann-Whitney U-test [26].

2.3. *Determination of water-soluble inositol phosphates*

AM monolayers were prelabeled for 40–45 h with 15–20 μCi/ml *myo*-[2-^{3}H(N)]inositol (15 Ci/mmol, NEN, Boston, MA) in 1 ml of inositol-free RPMI 1640 (modified RPMI) supplemented with 25 mM Hepes (pH 7.4), 3% heat-inactivated calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin.

After labeling, cell cultures were washed 3 times with Hepes-buffered modified RPMI 1640. The cells were then incubated in 1 ml of nonradioactive serum-free medium at 37°C with or without 20 mM LiCl, which is known to be an inositol phosphate phosphatase inhibitor [27]. FMLP (10^{-7} M) was added to the incubation mixture at the indicated times (from 1 to 10 min) before the end of the experiments. Reactions were stopped 40 min after LiCl addition by adding 2 ml of ice-cold 5% perchloric acid. [^{3}H]Inositol phosphates were then separated and quantified by anion-exchange chromatography of the aqueous phase according to Berridge et al. [28], and the radioactivity was determined by scintillation counting. Control studies using a labeled standard showed that elution yielded 95% recovery of IP_1. This method unequivocally separates inositol mono-, bis- and trisphosphates (IP_1, IP_2, IP_3) but does not distinguish their various possible isomers. The results are expressed as dpm of [^{3}H]inositol incorporation/10^6 AM (mean ± SE of three experiments).

3. RESULTS

The cell yield per ml of recovered bronchoalveolar fluid was equivalent in the 2 groups. Total cell counts averaged 140 ± 20 and 135 ± 9 × 10^3 in HS and AA, respectively. The percentages of AMs did not differ significantly between the 2 groups, and averaged 90 ± 2 and 80 ± 5 in HS and AA, respectively. Cell viability determined before culturing and experimentation averaged 95 ± 5%. In monolayer cultures, the adherent cells represented more than 90 ± 5% of the total cells based on latex phagocytosis. AM viability in cell cultures and just after [^{3}H]inositol labeling was greater than 85% as assessed by LDH released into the medium (<4 U/ml).

3.1. *Superoxide anion generation*

The stimulation of AMs by FMLP (10^{-7} M) resulted in an increase in O_2 release in both AA and HS. The increase was greater in AMs from AA than in those from HS. The difference between the two groups was slightly significant ($p < 0.05$) when the measurement was performed 1 min after FMLP stimulation. After 4 and 10 min, the O_2^- releases were 1.7 ± 0.4 vs 7.3 ± 1.3 and 1.1 ± 0.4 vs 4.9 ± 1.2 nmol/10^6 cells for AMs from HS and AA, respectively. The levels were significantly higher in AMs from AA than in those from HS ($p < 0.02$; fig.1).

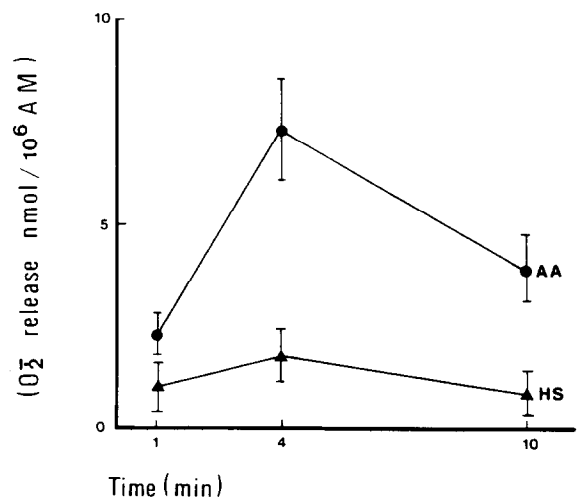

Fig.1. Superoxide anion release by FMLP-stimulated AMs from HS and AA. The results are the means ± SE of triplicate experiments.

3.2. *Inositol phosphate release*

Fig.2 shows the recovery of radioactive inositol phosphates in AM from HS in the presence or absence of LiCl. LiCl had no effect by itself on any of the radioactive inositol phosphate basal levels. Specifically, no significant difference was observed between the radioactivity incorporated into IP_3 in the presence or absence of LiCl (15000 ± 1130 vs 16600 ± 460 dpm, respectively). The stimulation by FMLP of these prelabeled quiescent macrophages induced a rapid release of IP_3, IP_2, IP_1 in the presence of LiCl lasted at least 10 min. For example, the addition of 10^{-7} M FMLP to the incubation medium produced a 2.7-, 2.25- and 2.75-fold increase in the accumulation of labeled IP_1, IP_2 and IP_3, respectively, after 10 min. The accumulation of inositol phosphates was rapid, i.e. it was already observed within 1 min of incubation, at which point the increase was mainly recovered in IP_3 (+ 50%). After a 4-min incubation of AMs with FMLP in the absence of LiCl, the elevated labeling of IP_1 returned to basal values. On the other hand, the increase in radioactive IP_2 and IP_3 induced by FMLP could be observed in the absence of LiCl.

The recovery of radioactive inositol phosphates in AMs from AA in the presence or absence of LiCl is shown in fig.3. The presence of 20 mM LiCl alone in the incubation mixture promoted a nearly 2-fold increase in the radioactivity in IP_1 (18700 ± 700 dpm in the presence of LiCl vs 10220 ± 520 dpm in the absence of LiCl) but had no significant effect on the basal activity in IP_2 and IP_3. Stimulation of these prelabeled cells by FMLP (10^{-7} M) in the presence of LiCl induced a further significant release of radioactive IP_1 but the effect was of low amplitude and was significant only after 10 min. IP_1 release was completely Li^+-dependent, i.e. in the absence of LiCl, cellular

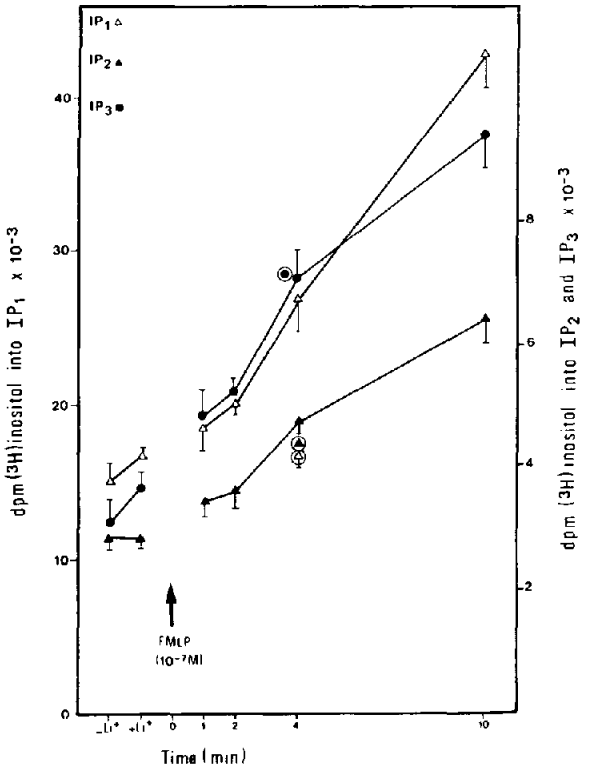

Fig.2. Accumulation of [^{3}H]inositol phosphate in AMs from HS before and after stimulation by FMLP. FMLP was added to cells that had previously received 20 mM LiCl. A control without LiCl (circled points) was done during stimulation with FMLP. The amount of label present in each inositol phosphate is expressed as dpm/10^6 cells. Each point represents the mean ± SE of 3 determinations in a single experiment.

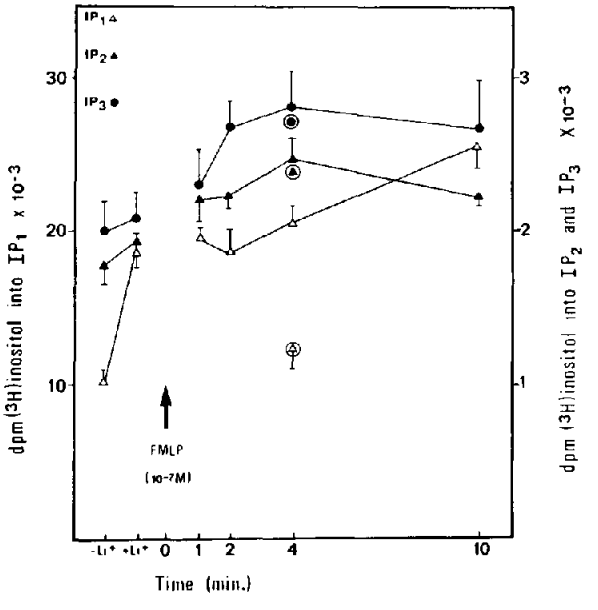

Fig.3. Accumulation of [^{3}H]inositol phosphates in AMs from AA before and after stimulation by FMLP. FMLP was added to cells that had previously received 20 mM LiCl. A control without LiCl (circled points) was done during stimulation with FMLP. The amount of label present in each inositol phosphate is expressed as dpm/10^6 cells. Each point represents the mean ± SE of 3 determinations in a single experiment.

radioactive IP_1 levels were the same as in controls after 4 min of FMLP treatment. Similarly, FMLP promoted only a slight increase in radioactive IP_2 and IP_3. These increases were much smaller than those observed in AMs from HS. No difference was observed between the basal values and the levels after 1 min. IP_2 and IP_3 increased after 4 min of incubation, at which point the largest increase was recovered in IP_3 (30% more than the basal value) and then decreased until 10 min. The level of labeled IP_1 returned to basal values in the absence of LiCl, and the increase in radioactive IP_2 and IP_3 was unchanged.

4. DISCUSSION

The present study shows that there are major differences in the accumulation of labeled inositol phosphates in the two kinds of AMs. For instance, we found that in AMs from HS, addition of LiCl, which blocks the breakdown of inositol phosphates [27], had no effect by itself on any of the radioactive inositol phosphate levels, indicating that phosphatidylinositol and the polyphosphoinositides are not metabolized at significant rates in these cells. On the other hand, in AMs from AA, there was a nearly 2-fold increase in labeled IP_1 when LiCl was added. This significant increase ($p < 0.001$) indicates that this inositol phosphate was spontaneously hydrolyzed by an Li^+-dependent IP_1 phosphatase. Since the presence of LiCl had no effect on the basal values of IP_2 and IP_3 in AM, the IP_1 accumulation in the presence of Li^+ observed in AMs from AA, could be exclusively the result of phosphatidylinositol breakdown. AMs from AA showed a continuous Li^+-sensitive production of IP_1 without any stimulation. This finding suggests that the cells from AA had been activated or 'primed' by in vivo exposure to inflammatory agents.

Addition of FMLP to the incubation medium produced very different effects on the release of inositol phosphates in AMs from the two populations. In AMs from HS, the accumulation of IP_1, IP_2 and IP_3 increased significantly ($p < 0.01$). The accumulation of IP_3 was large, and occurred as soon as 1 min after stimulus addition, indicating that the primary action of FMLP was to stimulate the hydrolysis of phosphatidylinositol bisphosphate yielding DAG and IP_3. After 4 min of stimulation by FMLP in the absence of LiCl, the elevated labeling of IP_1 returned to basal values, implying that the degradation of IP_1 by inositol 1-phosphate monophosphatase was very rapid and highly sensitive to Li^+, which is consistent with the inhibition of IP_1 phosphatase by Li^+ reported previously by Drummond et al. [29]. In contrast, addition of FMLP to AMs from AA usually promoted a small increase, if at all, in labeling IP_1, IP_2 and IP_3. The IP_1 release, observed after a lag phase, was much lower than in AM from HS. L'Allemain et al. [30] have shown that the formation of inositol phosphates in G_0-arrested fibroblasts is quite abundant compared to growing cells stimulated with thrombin, suggesting that the PPI pool available to phospholipase C is very small in the latter cells because of persistently stimulated PPI breakdown. The continuous production of IP_1 in the absence of stimulation in AM from AA, associated with the low formation of inositol phosphate following FMLP stimulation, implies that these cells were probably primed in vivo. The permanent activation of these cells leads to a depleted PPI pool, which explains the low value of inositol phosphate observed upon stimulation.

These results were also correlated with those obtained by measuring superoxide anion release during FMLP stimulation, showing an increase in this release by AM. The amounts of $O_2^{\cdot -}$ released by AMs from AA were significantly higher than those released by AM from HS. After 2 min, $O_2^{\cdot -}$ release by AMs from AA was 1.8-fold greater than by those from HS, and after 4 min, it was 4.2-fold greater. These higher responses of AMs from AA could also be due to a 'priming' process characterized by an enhancement of stimulated $O_2^{\cdot -}$ release following in vivo exposure to inflammatory agents [31]. Moreover, Johnston et al. [32] have shown that priming for an enhanced respiratory burst is an essential component of macrophage activation, which is the principal end result of cell-mediated immunity.

Thus, the results reported here suggest that the modifications of the two parameters studied in vitro arose from in vivo cellular activation. This could explain the results reported by Hoidal et al. [33] and Greening et al. [34] in asymptomatic cigarette smokers, by Clement et al. [35] in children's interstitial lung disease, who found that activated AMs released more $O_2^{\cdot -}$ than AMs from

control subjects, and by Holian et al. [36] followed by Rossman et al. [37], who found that AMs from guinea-pigs could be stimulated by N-formyl-methionyl-peptides to produce O_2^-. Formyl peptide-stimulated O_2^- production by these AMs has been shown to occur through receptor activation, and is associated with stimulated turnover of phosphatidyl inositols [15,38]. Our results obtained in human AMs are in agreement with such a mechanism, and more specifically show that AMs from AA were continuously activated, clearly indicating that these cells were primed in vivo by mediators present at the inflammatory sites.

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