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Adhesive interactions of neutrophils and leukotriene synthesis

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Cell–substrate and cell–cell adhesion of neutrophils has been found to slow down the calcium ionophore A23187-induced synthesis of 5-lipoxygenase (5-LO) metabolites of arachidonic acid. Addition of the exogenous substrate, arachidonic acid (AA), together with A23187, resulted in the enhanced production of leukotriene B4 (LTB4) and 5-hydroxyeicosatetraenoic acid (5-HETE) by adherent neutrophils in comparison with those by the cells in suspension. We observed also the enhanced production of 5-LO metabolites in attached cells when we stimulated the cells by the combined action of phorbol 12-myristate 13-acetate (PMA) and A23187. Thus, the adhesion to solid substrate and to other cells, an important regulatory factor for the activity of many cells, is a powerful regulator of leukotriene production by neutrophils.

5-Lipoxygenase; Leukotriene synthesis; Adhesion; Human neutrophil

1. INTRODUCTION

Neutrophils are the blood cells for which adhesion is one of the main functions. Recent studies have demonstrated that neutrophils adhered to biological surfaces show different characteristics of activation compared with neutrophils in suspension [1,2], in particular, the respiratory burst in suspended neutrophils in response to formyl-methionyl-leucyl-phenylalanine is short term, and the O_2^- production is considerably lower compared with the cells attached to the surface [2].

One of the functional responses of neutrophils to stimuli is the activation of a 5-lipoxygenase (5-LO) cascade and the synthesis of a potent chemotactic agent, leukotriene B4 LTB4, and other products. Several recent lines of experiment have directly demonstrated that adhesion alters arachidonic acid (AA) metabolism in phagocytes [3,4]. Brady and Serhan [4] have found that adhesion promotes trans-cellular leukotriene biosynthesis during neutrophil–glomerular endothelial cell interactions. Leukotriene generation was inhibited in the presence of antibodies against CD11/CD18 integrins and L-selectin. In previous studies, Kouzan et al. [3] demonstrated that macrophages adhered to plastic exhibited a dramatic shift towards the lipoxygenase pathway compared with the metabolic profile of cells treated with the calcium ionophore, A23187, in suspension.

In the present work we investigated the role of cell–substrate and cell–cell contacts in neutrophil leukotriene synthesis. We have found that after attachment of neutrophils to glass surfaces, when numerous cell–cell contacts have been established, the A23187-stimulated synthesis of the 5-LO metabolites is attenuated. When the exogenous substrate, AA is added together with A23187, the 5-LO metabolite production in adherent cells is higher than in suspended cells. When we stimulated neutrophils by the combined action of PMA and A23187, the synthesis of 5-LO metabolites by adherent cells was greatly enhanced in comparison to that by the cells in suspension.

2. MATERIALS AND METHODS

2.1. *Media*

Dulbecco's phosphate-buffered saline (PBS), Hank's balanced salt-solution (HBSS) without Phenol red and bicarbonate were from Sigma. Ficoll-Paque and Dextran T-500 were from Pharmacia; 2′,7′-bis(2-carboxymethyl)-5(6)carboxy-fluorescein acetoxymethyl ether (BCECF-AM) was from Molecular probes; AA, A23187, triethylamine were from Sigma; dimethylsulfoxide and trifluoroacetic acid (TFA) were purchased from Merck; Sep-Pak was from Waters; and methanol was from Reachim (Russian Federation).

2.2. *Neutrophils*

Neutrophils were isolated from freshly drawn donor blood on a bilayer gradient of Ficoll-Paque (1.077 and 1.125) [5]. Washed neutrophils were resuspended in bicarbonate-free HBSS containing 10 mM HEPES, pH 7.4.

2.3. *Leukotriene synthesis by adhered cells*

A suspension of neutrophils (2–7 × 10^6/ml, was placed into a Karrel glass vessel in a volume sufficient to fit its wall surface, and was incubated at 37°C for 20–30 min. It was seen under the microscope that more than 90% of cells adhered to the glass. The cells were then stimulated for 5 min at 37°C with A23187 (5 μM) or A23187 plus PMA (100 nM) or A23187 plus 30 μM AA with 0.01 μCi/ml [^{14}C]AA.

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Abbreviations: 5-LO, 5-lipoxygenase; AA, arachidonic acid; LTB4, leukotriene B4, 5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; 5-HETE, 5*S*-hydroxy-6,8,11,14-eicosatetraenoic acid; *trans*-LTB4 isomers, 5*S*,12*R*-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid and 5*S*,12*S*-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid.

The synthesis was stopped by 1 vol. of methanol. Prostaglandin B_2 was the internal standard.

2.4. *Leukotriene synthesis in suspension*

A neutrophil suspension of the same cell concentration as in the experiment with adhered cells was incubated in a siliconized glass vessel at 37°C with shaking and stimulated as described above.

2.5. *Intracellular pH measurement*

Cells were loaded with the fluorescent dye, BCECF, by incubation with its acetoxymethyl ether (BCECF-AM) according to [6]. Measurements on a single cell basis were performed according to the method of Paradiso et al. [7] using a Zeiss photomicroscope III (Germany) with a microfluorometer (LOMO). Calibration of the fluorescent signal was performed by a nigericin/KCl clamping method [8]. For pH_i measurements in the suspension, a Jobin Yvon spectrofluorimeter with a thermostatic cuvette and stirring was used. Calibration was achieved by killing cells with 0.1% Triton X-100 [6].

2.6. *$^{45}Ca^{2+}$ influx into neutrophils*

The influx induced by A23187 was measured in suspended and adhered cells. A tracer amount of $^{45}CaCl_2$ (10 μCi/ml of cells) was added to cells simultaneously with 5 μM A23187. After 5 min incubation supernatant was withdrawn, 0.5 ml 2% SDS was added to cells, and their radioactivity was determined by a liquid scintillation method.

2.7. *Lipoxygenase products*

These were separated by RP-HPLC. The denatured cell suspension was centrifuged at 1,000 × *g* (4°C, 15 min), and the supernatant was purified by solid-phase extraction on C18 Sep-Pak (Waters) as described elsewhere [9]. The purified samples were injected on to an Altex C18 5 μm column (250 × 4.6 mm). The products were eluted at 1 ml/min in a gradient mode: 0–30 min linear gradient from 20 to 90% B; 30–35 min linear gradient from 90 to 100% B. Eluents consisted of methanol/water/TFA triethilamine in the ratio (A) 50/50/0.1/0.025, (B) 100/0/0.1/0.025. Elution was monitored using Jusco (Japan) model 875-UV detector (280 nm, 0–23 min; then 235 nm) and LKB-Wallac 1208 Betacord Radioactivity Monitor when [^{14}C]AA was added. The reaction products were quantitated by comparison of peak areas with the internal standard, prostaglandin B_2. The lower limit of detection was 5 ng at 280 nm and 10 ng at 235 nm.

3. RESULTS AND DISCUSSION

We measured the formation of the following products of 5-LO oxidation of AA: LTB4, its ω-oxidized metabolites, 6-*trans*-LTB4 isomers, and 5-HETE. A chromatogram of the 5-LO metabolites is presented in Fig. 1.

Table I

Leukotriene synthesis by attached and suspended neutrophils stimulated by 5 μM of the calcium ionophore, A23187

Synthesis by	nmol/10^7 cells			
	ω-Oxidized LTB4	LTB4	All-*trans* LTB4	5-HETE
Attached cells	0.16 ± 0.02	0.33 ± 0.04	0.12 ± 0.01	0.80 ± 0.09
Cells in suspension	0.37 ± 0.04	0.50 ± 0.06	0.42 ± 0.05	1.2 ± 0.13

Cell concentration = 2–7 × 10^6/ml in suspension and 1,100–4,700/mm^2 in Karrel glass, 5 min incubation.

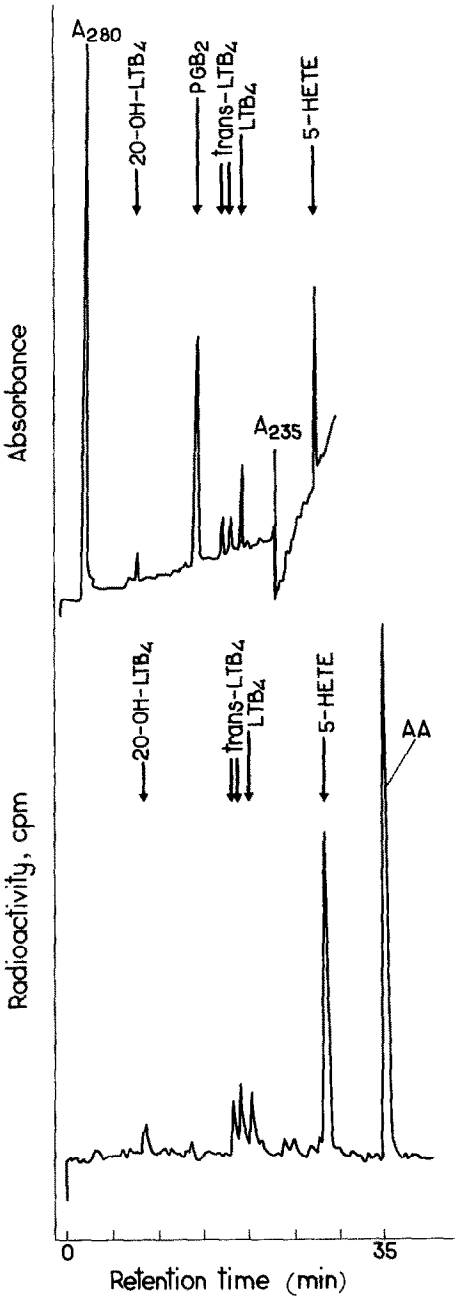

Fig. 1. 5-LO products formed by adherent human neutrophils exposed to 5 μM A3187 with 30 μM AA, monitored for UV absorbance (280 nm, 0–23 min; then 235 nm) and for radioactivity. The retention times of the authentic standards are indicated.

The suspended cells do not synthesize 5-LO products of AA without stimuli. Neither the adhesion of neutrophils to the glass nor neighboring cells per se initiates the 5-LO cascade in neutrophils, unless the cells were stimulated. However, adhesion decreases the leukotriene synthesis in calcium ionophore A23187-stimulated neutrophils (Table I). The slowdown of the synthesis by adherent cells occurs both during long-term (30 min, data not shown) and short-term (5 min) incubation, i.e. it appears to be related to the decreased production of

Table II

Dependence of intracellular pH on cell density

Cell density	Cells/mm^2	pH_i
1. Single cells, no contact with each other	312	6.85 ± 0.02
2. Cells, forming groups of 3–5 contacting cells	1,755	7.08 ± 0.02
	2,262	7.12 ± 0.03
3. Cells, forming a monolayer	4,446	7.23 ± 0.03

These data present the results of one typical experiment. Mean values of pH_i ± S.E.M. are indicated. The difference in pH_i between different zones is statistically significant ($P < 0.05$).

Table III

Comparison of the amount of LTB4 (nmol/10^7 cells) synthesized in suspended neutrophils under different pH and ionophore concentrations

A23187 (μM)	pH = 7.2 (pH_i = 7.16*)	pH = 7.8 (pH_i = 7.27*)
0.1	0.087 ± 0.012	0.16 ± 0.02
0.6	0.42 ± 0.05	0.51 ± 0.06
3	0.41 ± 0.06	0.75 ± 0.06
9	0.55 ± 0.08	0.99 ± 0.09

Neutrophils were stimulated by indicated concentrations of the calcium ionophore, A23187. Cell concentration = 8.8×10^6/ml, 1.5 min incubation.

*pH_i in suspension was measured as described in section 2.

the 5-LO metabolites rather than to the change of the process kinetics.

What kind of factors can account for the differences in leukotriene synthesis between adhered and suspended cells?

It is known that leukotriene synthesis in human neutrophils requires (i) the release of endogenous substrate and (ii) activation of 5-LO. High levels of cytosolic free Ca^{2+} [10], along with a sustained Ca^{2+} influx across the plasma membrane into the cell [11], are the key elements required for the activation of 5-LO, while the enzymic activity is known to be further modulated by intracellular pH [12].

In our previous work we have found that intracellular pH (pH_i) depends on the contacts the cell has established with the neighboring cells and the substrate [13,14]. It was shown that adhesion to glass surfaces resulted in a pH_i increase of about 0.2 units [13]. The difference in cell density results in an upward pH_i shift of about 0.3 units in fibroblasts [14] and 0.4 units in neutrophils (Table II). These results show that the effect of adhesion on the intracellular pH is favorable for leukotriene synthesis. Moreover, when we simulated the upward ΔpH_i shift in suspended cells by changing of extracellular pH, we found that in the ionophore-stimulated suspended cells the increase in pH_i leads to acceleration of leukotriene synthesis (Table III).

We have also found that the attachment of neutrophils to the glass surface and to neighboring cells increases Ca^{2+} influx into cells. Experiments addressing ^{45}Ca influx in response to A23187 (measured as described in section 2 revealed an increase in the ^{45}Ca equal to 2,000 ± 180 cpm/6×10^6 cells in suspended cells and 5,270 ± 300 cpm/6×10^6 cells in the adherent ones, i.e. the effect of adhesion on the intracellular Ca^{2+} concentration is also favorable for leukotriene synthesis. In previous studies it was also shown that integrin-mediated adhesion increases the cytosolic free Ca^{2+} concentration in monocyte cells through the release of Ca^{2+} from the intracellular stores as well as Ca^{2+} influx from the external compartment [15].

The limiting factor for leukotriene synthesis is the availability of the endogenous substrate, AA. The AA availability may be different in suspended and adhered cells. It has been shown that AA mobilization is attenuated when neutrophils are activated in suspension at high cell density [16]. Neutrophils in suspension undergo adhesion with the formation of neutrophil–neutrophil aggregates upon stimulation. One may suppose that prolonged contact between adherent cells can markedly affect AA liberation. In our experiment, the addition of exogenous AA together with A23187 to suspended and adherent neutrophils revealed that the addition of exogenous AA abolishes adhesion-induced inhibition of leukotriene synthesis, and that the formation of 5-LO metabolites of AA in adherent cells is higher than in suspended cells (Table IV). We have determined the specific radioactivity of the products when AA of known specific radioactivity was added simultaneously with the ionophore to suspended and adherent cells, and

Table IV

Comparison of the synthesis of 5-LO metabolites in attached and suspended neutrophils induced by the calcium ionophore, A23187 (5 μM), and 30 μM AA with 0.01 μCi/ml [^{14}C]AA

Synthesis by	nmol/10^7 cells				Specific radioactivity (cpm/μg)	
	ω-Oxidized LTB4	LTB4	All-*trans* LTB4	5-HETE	LTB4	5-HETE
Attached cells	0.14 ± 0.02	1.0 ± 0.1	1.4 ± 0.15	7.7 ± 1.8	1,400 ± 100	1,700 ± 200
Cells in suspension	0.25 ± 0.04	0.62 ± 0.07	1.4 ± 0.2	4.9 ± 1.1	830 ± 100	1,200 ± 140

Cell concentration = 2–6 × 10^6/ml in suspension and 2,500–3,100/mm^2 in Karrel glass, 5 min incubation.

Table V

Comparison of the synthesis of 5-LO metabolites in attached and suspended neutrophils induced by the calcium ionophore, A23187 (5 μM), and 100 nM PMA

Synthesis by	nmol/10^7 cells			
	ω-Oxidized LTB4	LTB4	All-*trans* LTB4	5-HETE
Attached cells	0.10 ± 0.02	0.50 ± 0.06	0.23 ± 0.03	1.4 ± 0.2
Cells in suspension	0.18 ± 0.02	0.29 ± 0.04	0.09 ± 0.02	0.65 ± 0.08

Cell concentration = 3–7 × 10^6/ml in suspension and 4,200–5,500/mm^2 in Karrel glass, 5 min incubation.

Table VI

Effect of adhesion on formation of 5-LO products by neutrophils

Stimuli	Synthesis by	
	Cells in suspension	Attached cells
1. A23187 (5 μM)	100 ± 11	56 ± 6
2. A23187 (5 μM) + AA (30 μM)	100 ± 15	146 ± 18
3. A23187 (5 μM) + PMA (100 nM)	100 ± 14	183 ± 20

The sum of the 5-LO metabolites produced by adherent cells is shown as a percentage of those formed by suspended cells. For this table the data from Tables IV and V have been used.

found (Table IV) that the specific radioactivity of LTB4 and 5-HETE is increased in adherent cells in comparison with the suspended ones. It indicates that in adhered neutrophils the synthesis results substantially from the exogenous substrate.

It is known that phospholipase A_2 plays the major role in the liberation of AA. According to recent findings [17], PMA can modulate the A23187-stimulated AA mobilization so as to render it less sensitive to inhibitors of phospholipase A_2. We have found that under the combined action of PMA and A23187, in the absence of exogenous AA, the formation of 5-LO metabolites in adherent neutrophils is more effective than in suspended neutrophils (Tables V and VI).

Thus, the adhesion to solid substrata and to other cells, an important regulatory factor for the activity of many cells, is the powerful regulator of leukotriene production by neutrophils. After attachment of neutrophils to a solid surface and to neighboring cells the synthesis of the 5-LO metabolites is attenuated. The inhibition of the 5-LO synthesis in the adhered cells is caused by the decreased availability of endogenous AA, and in the presence of exogenous substrate the synthesis of 5-LO metabolites in adherent cells is even higher than in suspended neutrophils. The increase in the 5-LO activity is in agreement with the adhesion-stimulated increase of the H^+ and Ca^{2+} intracellular concentrations which are critical for 5-LO activity.

Adhesive interactions of cells with each other and non-cellular substrates are regulated by membrane molecules called integrins [18–20]. The occupancy of integrins after cell attachment represents a signal which may interact with the pathways of signal transduction in activated neutrophils. The mechanism by which adhesive receptors display these effects are far from being elucidated. Quite recently it was found [21] that platelet integrin glycoprotein IIb-IIIa is associated with a 21-kDa GTP-binding protein. It is known that G-proteins are involved in phospholipase A_2 activation [22]. Adhesive contacts might reduce leukotriene synthesis via G-protein-mediated inhibition of phospholipase A_2. Exogenous AA abolishes the effect of such an inhibition by drawing off the exogenous substrate into the reaction. In turn, addition of PMA, which favors enhanced attachment of cells to a solid surface, may also prevent the inhibition of phospholipase A_2 and hence activate 5-LO in adherent neutrophils.

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