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PRELIMINARY COMMUNICATION

ASSOCIATION OF SODIUM SALICYLATE TO ISOLATED LEUCOCYTES

Maikel Raghoebar*, Harry L.G.M. Tiemessen*, Pierre A.M. Peeters*, Wim B. van den van den Berg**, Cees A.M. van Ginneken*, Levinus B.A. van der Putte**

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* Department of Pharmacology, University of Nijmegen, P.O.B. 9101, 6500 HB Nijmegen. The Netherlands

** Department of Rheumatology, University Hospital, St. Radboud, Nijmegen. The Netherlands.

It has been established from the work of Brune and co-workers (1-5) that acidic non-steroidal anti-inflammatory drugs (NSAIDs) reach higher concentrations in inflamed tissues than in most others throughout the body. However, it is still unknown which pharmacokinetic events are leading to accumulation of NSAIDs in inflamed areas. The present study is based on the hypothesis that leucocytes are mediators of the anti-inflammatory activity of drugs, both as target cells and as potential vehicles for the transport of anti-inflammatories to inflamed tissues. The transport of these drugs via binding on the surface and/or granulocyte internalization is of interest, since an early sign of inflammation usually is an influx of polymorphonuclear (PMN) leucocytes (6-8) and many of the mechanisms possibly involved in the action of NSAIDs are related to leucocytes (2, 9-14). In this study the association of sodium salicylate (SA) to PMN leucocytes is investigated.

Experimental Procedures

Materials

The radiolabelled compounds ^{14}C-salicylic acid (sp. act. 53.8 mCi/mmole) and ^{3}H-inulin (sp. act. 1.84 Ci/mmole) were purchased from New England Nuclear (Boston, USA) and the Radiochemical Centre Amersham (Utrecht, The Netherlands) respectively. Sodium salicylate from Merck (Darmstadt, FRG). Ficoll-paque (sp.g. 1.077), Dextran T-500 and Percoll (sp.g. 1.130) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Other chemicals were of reagent grade and obtained from Merck (Darmstadt, FRG).

Separation and characterization of PMN leucocytes

Density sedimentation. Fresh whole blood was obtained from healthy adult volunteers and fresh buffy coats (diluted 1:1 with phosphate buffered saline) were purchased from the local Blood Bank. Neutrophils were isolated by a combination of dextran sedimentation and Ficoll-paque cushion centrifugation (15) and Percoll (sp.g. 1.098) cushion centrifugation (15 min at 800 x g at 20°C) to remove the contaminating red cells. The interface was collected and washed several times with phosphate buffered saline. Finally the cells were resuspended in Hanks balanced salt solution.

Counterflow centrifugation. Elutriation according to De Mulder et al. (16) was performed with buffy coats, obtained after dextran sedimentation. Phosphate buffered saline was used as the elutriation

medium; the counter flow rate was 7.0 ml/min. The PMN leucocytes were suspended in Hanks balanced salt solution (10-15 x 10^6/ml).

Cell characterization. Cell number was counted microscopically in a Bürker chamber after staining with Türk reagent. Viability as determined by trypan blue exclusion was approximately 95-98% for both isolation methods. Cyto-centrifuge slides were made and stained with May Grünwald-Giemsa. Microscopic examination of leucocyte preparations showed approximately 96% of PMN and 4% of lymphocytes, monocytes and eosinophilic granulocytes and absence of erythrocytes.

Ligand-binding assays

Silicone oil cushion method. Binding of sodium ^{14}C-salicylate to PMN leucocytes was determined by a modified centrifugation technique. An aliquot of a cell suspension containing 3 x 10^6 PMNs, ligand and Hanks balanced buffer in a total volume of 600 µl were layered on top of a 100 µl cushion of silicone oil (Wacker Chemie, München, FRG) with a density of 1.040 kg/m^3. Incubation was performed in an Eppendorf microtube at 37°C or 4°C during 1 hr. Cells were separated from medium by centrifugation for 5 min at 14000-18000 g in a Heraeus Hämofuge. The tip of the microtube was excised and the pellet was solubilized with 1.0 ml Soluene-100 (Packard Benelux, Brussels, Belgium) and mixed with 13 ml of Instagel-1 N HCl (9:1) (Packard). Under the experimental circumstances the silicone oil fraction appeared to be free of SA.

Double centrifugation method. Binding experiments with decayed or lysed cells or experiments in which the cells lost their intactness during the incubation period, could not be performed with the silicone oil cushion method. The lower density of the membrane fragments prevented sedimentation through the oil. Hence, in these experiments the oil was omitted and after centrifugation the supernatant was separated from the pellet manually by retrograde centrifugation.

Results and Discussion

Characterization and viability of leucocytes during incubation with SA

During the incubation period of 1 hr with SA concentrations of 0-60 µmole/ml at 37°C the cell count and the viability did not change. With higher SA concentrations the viability of the PMN leucocytes decreased.

Cell association of SA to PMNs

The cell association of SA can be interpreted as a combination of nonspecific and specific (saturable) reversible mechanisms; there were only minor differences in the cell association between the PMN isolation methods (Figure 1A). Possible differences may be related to the fact that the dextran-Ficoll-paque-Percoll-method may be somewhat more harmful to the recovered PMN leucocytes than the elutriation method (17). The binding of SA to purified erythrocytes (10^6-10^7 cells/600 µl incubation medium) is very small. The overall percentage of SA cell association of 0.01-0.02 to 3 x 10^6 erythrocytes is about ten times lower than the cell association to 3 x 10^6 PMN leucocytes (0.1-0.2% overall binding). The binding of SA to a mixed fraction of lymphocytes and monocytes was definitely less (about 0.05-0.1% cell-associated SA) than to PMN leucocytes.

Effect of temperature on cell association of SA to PMNs

The cell association of SA is significantly reduced by decreasing the incubation temperature from 37°C to 4°C (Figure 1B), but is not totally inhibited. This observation suggests that cell association of SA probably not only occurs by nonspecific mechanisms such as fluid phase endocytosis but also by more specific mechanisms such as adsorptive endocytosis (18, 19).

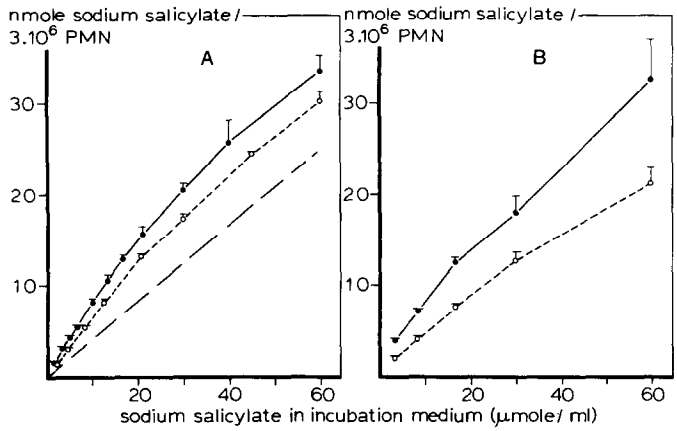

Fig. 1 A. Concentration of sodium salicylate in incubation medium versus cell associated sodium salicylate.
(•) PMN isolation by density sedimentation; (o) PMN isolation by elutriation;
(- -) nonspecific cell association as estimated from the terminal linear part of the binding curves

B. Effect of temperature on cell association of sodium salicylate
(•) incubation at 37°C; (o) incubation at 4°C; PMN isolation by density sedimentation
Binding assay: silicone oil cushion method. Presented are the mean values ± SD of three experiments.

Effect of the number of PMNs on cell association of SA

There is a linear relationship between the amount cell-associated SA and the number of PMN leucocytes from 3 x 10^6 cells/600 µl incubation medium (Figure 2A). For this reason it is decided to use minimal cell concentrations of 3 x 10^6/600 µl. The degree of cell association of the extracellular marker and marker for fluid phase endocytosis ^{3}H-inulin is constant in experiments performed by increasing the number of PMN and is comparable with the degree of cell association of SA to low PMN concentrations (about 0.05% cell association of SA). Comparison of the binding curves for 3.3 µmole/ml and 16.7 µmole/ml SA provided a larger amount of cell association for 16.7 µmole/ml, but a higher percentual degree of cell association for 3.3 µmole/ml SA (Figure 2B). It appears that the cell association of

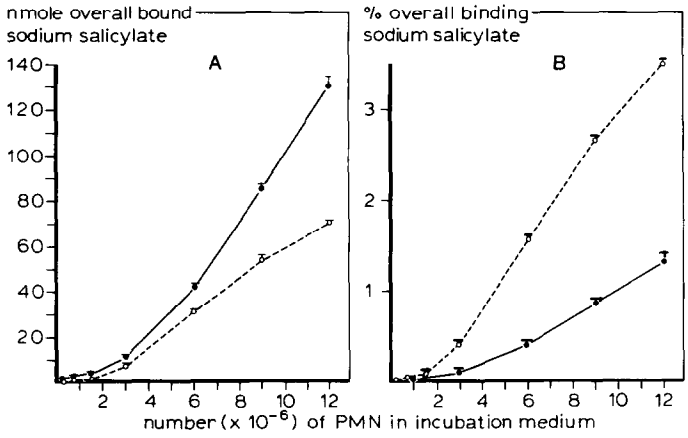

Fig. 2 Effect of the number of PMN on cell association of sodium salicylate at 37°C
(o) 3.3 µmole of SA per ml incubation medium; (•) 16.7 µmole of SA per ml incubation medium.
A. absolute amount of SA bound
B. percentual binding of SA
PMN isolation by density sedimentation; Binding assay: silicone oil cushion method.
Presented are the mean values ± SD of three experiments.

SA to intact PMNs is saturable. This is also in accordance with the idea that the cell association at least partly occurs by adsorptive endocytosis of ligands which bind to acceptors on the cell membrane.

Effect of decay of PMNs on the association of SA

Association of SA to homogenized cell suspensions was more extensive than to the same volume of intact cells, even at 4°C (Figure 3). These findings can be explained by pronounced intracellular binding sites for SA or enhanced binding by uptake by vesicles formed during lysis of the cells.

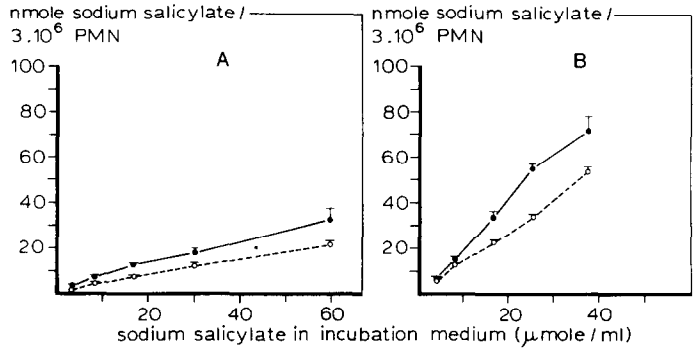

Fig. 3 Effect of decay of PMN on cell association of sodium salicylate
(•) incubation at 37°C; (o) incubation at 4°C.
A. Intact cells (binding assay: silicone oil cushion method)
B. Lysed cells (binding assay: double centrifugation method)
PMN isolation by density sedimentation.
Presented are the mean values ± SD of three experiments

Conclusions

The silicone oil cushion method appears to be an elegant and reproducible method to separate cell associated from unassociated ligand (20, 21, 22). The cell association of SA can be interpreted as a combination of nonspecific and specific (saturable) reversible mechanisms. Cell association of SA tends to increase when lysis of the PMNs occurs and is temperature-dependent. Further research on the mode of cell association of SA to intact and lyzed PMN leucocytes and cell association of other NSAIDs to these cells is in progress.

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