Sulphasalazine inhibition of human granulocyte activation by inhibition of second messenger compounds

Gunnar Carlin, Richard Djursäter, Göran Smedegård

Abstract

The effects of sulphasalazine on the production of second messenger compounds in human granulocytes have been characterised by various stimuli. The increase in cytosolic calcium, inositol triphosphate, diacylglycerol, and phosphatidic acid (all important mediators of intracellular signal transduction) triggered by stimulation were inhibited by sulphasalazine. The metabolites 5-amino-

Sulphasalazine, a drug used for the treatment of inflammatory bowel disease,1 has during the last decade also been shown to treat effectively other diseases of a presumably autoimmune nature, such as rheumatoid arthritis, ankylosing spondylitis,2 and psoriasis.3

The aetiology of tissue injury in rheumatoid arthritis is unclear. A variety of cells, including granulocytes, have been suggested to contribute to the initiation and propagation of the immune response in the rheumatoid synovium. Kitis and Weissmann4 have reviewed the role of the neutrophil granulocyte as a key player in the articular and extra-articular manifestations of rheumatoid arthritis. They compared rheumatoid joint inflammation to the Arthus reaction, a neutrophil dependent interaction in which repeated exposure to an antigen results in the formation of immune complexes and activation of complement, causing an influx of granulocytes and tissue destruction.

Sulphasalazine has been found to inhibit granulocyte activation, assessed as superoxide production by cells activated with serum treated zymosan,5 chemotactic peptide N-formyl-t-leucyl-l-leucyl-l-phenylalanine (fMLP)6 7 and immune complexes.8 Sulphasalazine has also been found to inhibit, for example, granulocyte chemotaxis,9 lymphocyte proliferation,10 and interleukin 2 production11 in addition to monocyte production of interleukin 1.12

We have previously found by using the granulocyte as a model system that sulphasalazine inhibits cellular activation before activation of protein kinase C by interference with the synthesis of phosphoinositide derived second messenger compounds.5 As several cell types utilise phosphoinositide dependent signal transduction, sulphasalazine may modify a principal common mechanism for the regulation of cell activity, which may explain the beneficial effects of the drug in a variety of diseases.

Many of the stimuli used in the earlier studies trigger an increase in cytosolic calcium as part of the activation sequence. The increase in calcium has, in a variety of cell systems, been found to depend on remodelling of phosphoinositides13 (see fig 1). Ligation of a cell receptor leads to the phospholipase C dependent hydrolysis of phosphatidylinositol-4,5-bisphosphate generating two triggers of cell responses, namely diacylglycerol, an activator of protein kinase C,14 and inositol-1,4,5-trisphosphate, which triggers the release of cell associated calcium.13 The link between the receptor and phospholipase C is in many cells a guanosine 5'-trisphosphate (GTP) binding protein.15

Several drugs have been shown to modulate granulocyte responses such as superoxide production,16 and a few studies have indirectly associated the drug action with an interaction with the synthesis of phosphoinositide derived second messenger molecules17 18 and the GTP binding protein.19

The purpose of this study was to evaluate whether the inhibitory effects of sulphasalazine on cellular activation were related to the production of second messenger compounds. We found that sulphasalazine inhibited the production of inositol-1,4,5-trisphosphate and consequently the increase in cytosolic calcium, which indicated an interaction with either the plasma membrane receptor, the associated GTP binding protein, or the phospholipase C. Sulphasalazine still inhibited activation of cells stimulated with guanosine 5'-[γ-thio]triphos-

The production of phosphatidic acid, a metabolite of diacylglycerol, the other product of phospholipase C activity, was also inhibited by sulphasalazine in cells unspecifically labelled in the lipid glyceride backbone. Phosphatidic acid is alternatively formed by hydrolysis of phosphatidylcholine; we found that sulphasalazine also inhibited the production of radioactive phosphatidic acid in cells specifically labelled in phosphatidylcholine.

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**Materials and methods**

**TEST COMPOUNDS**

Sulphasalazine, sulphasalazine, and 5-aminosalicylic acid were from Kabi Pharmacia (Uppsala, Sweden).

**BUFFER**

Dulbecco’s phosphate buffered saline (PBS) without, or when specifically stated without, 0.9 mM Ca\(^{2+}\) and 0.5 mM Mg\(^{2+}\) was from Statens Veterinärmedicinska Anstalt (Uppsala, Sweden).

**ISOLATION OF GRANULOCYTES**

Granulocytes were isolated from heparinised blood from healthy volunteers, not using anti-inflammatory drugs, by the method of Boyum\(^{20}\) with minor modifications.\(^{6}\)

**INOSITOL-1,4,5-TRISPHOSPHATE SYNTHESIS**

Granulocytes, 1.5x10\(^6\)/ml, were labelled with 1110 kBq/ml of \(^{[\text{H}]\text{myo-inositol (TRK.856, Amersham, Solna, Sweden, for two hours at 37°C as described previously.}\(^{21}\) Labelled granulocytes were washed and resuspended in Hanks’ balanced salt solution (HBSS, Gibco, Paisley, UK) containing 10 mM LiCl. Each sample, containing 25x10\(^6\) cells in 1 ml HBSS with LiCl, was preincubated with the test compound for 10 minutes at 37°C before challenge with 0.1 uM fMLP (Calbiochem, La Jolla, CA, USA). The reaction was terminated by addition of 7.5% trichloroacetic acid. Inositol-1,4,5-trisphosphate was isolated by ion exchange chromatography as described previously.\(^{21}\)

**CYTOSOLIC CALCIUM**

The granulocytes, 1x10\(^8\)/ml, were incubated with 10 \(\mu\)M FURA-2AM (Molecular Probes Inc., Eugene, OR, USA) as described by Korchak et al.\(^{22}\) The FURA-2 loaded cells were suspended with 25x10\(^6\) cells/ml in ice cold PBS without Ca\(^{2+}\) and Mg\(^{2+}\) until used.

The cells, 2.5x10\(^6\)/ml were preincubated in PBS either with or without Ca\(^{2+}\) and Mg\(^{2+}\) (in the latter case the buffer was supplemented with 1 mM EGTA) and test compound for two minutes before the addition of stimulus. Fluorescence changes were monitored in a Shimadzu RF-5000 spectrophotometer equipped with a continuous magnetic stirring device at the dual excitation wavelengths of 340 and 380 nm, and emission at 510 nm. The concentration of cytosolic calcium was calculated by the ratio method of Grynkiewicz et al.\(^{23}\)

Sulphasalazine at 10 nM had considerable absorbances at 340 and 380 nm. As the reduction of the emitted light was about 25% at the two wavelengths, however, sulphasalazine had only a negligible effect on the calculations of [Ca\(^{2+}\)] using the ratio method.

**GTP INDUCED SUPEROXIDE PRODUCTION**

The granulocytes were electroporpermeabilised essentially as described by Nasmith et al.\(^{24}\) Cells were resuspended in permeabilisation buffer to 10\(^7\) cells/ml and permeabilised with two discharges of 5 kV/cm from a 25 \(\mu\)F capacitor by using a Bio-Rad gene pulser apparatus equipped with a 0.8 ml cuvette. Permeabilised cells were used immediately (within a few minutes). The permeabilisation buffer contained 10 mM HEPES, 140 mM KCl, 1 mM EGTA, and 0.193 mM CaCl\(_2\) (giving a free [Ca\(^{2+}\)] concentration of 100 nM), 1 mM MgCl\(_2\), and 10 mM glucose (pH 7.0, 295 mosM).

A 133 \(\mu\)M solution of cytochrome c (type VI from horse heart, Sigma Chemical, St. Louis, MO, USA) in 750 \(\mu\)L permeabilisation buffer containing the test compound was prewarmed to 37°C. Thereafter 100 \(\mu\)L of a 20 mM NADPH solution was added immediately followed by 50 \(\mu\)L of the cell suspension and 100 \(\mu\)L of GTP[S]. Prolonged exposure of NADPH to cytochrome c will result in the reduction of the latter and was thus avoided. After incubation at 37°C the absorbances at 550 and 540 nm were recorded spectrophotometrically.\(^{5}\) The superoxide production was expressed as the difference in absorbance at these wavelengths.\(^{5}\) All values...
given are the mean of at least duplicate parallel experiments.

**GTPase Activity**

Granulocytes were suspended in 0.25 M sucrose, 10 mM HEPES, and 1 mM EGTA (pH 7.5) to about 10^7 cells/ml. The cell suspension was sonicated with a 10 second pulse of a Branson B15 cell disruptor. Intact cells and nuclei were removed by 10 minutes of centrifugation at 400 g, and membranes were sedimented by centrifugation for 30 minutes at 16000 g. Membranes were either used immediately or frozen.

GTPase activity was calculated by measuring the liberation of phosphate-32 from [γ-32P]GTP as described by Pelz et al. The reaction mixture contained 10 µl cell membrane suspension (membranes from the granulocytes in about 3 ml blood), 100 mM buffer (100 mM NaCl, 20 mM TRIS-HCl, 5 mM MgCl₂, 0.1 mM EGTA, 1.14 mM ATP, 500 µM adenylylimidodiphosphate, 0.25 mM ouabain, pH 7.5) with about 50 nmol/added [γ-32P]-GTP (NEG-004, New England Nuclear, Stockholm, Sweden; 1-22 TBU/mmol radioactivity). The reaction was terminated after 10 minutes by addition of 0.5 ml of a 5% charcoal mixture containing 0.1% dextran 500 (Kabi Pharmacia) and 0.5% bovine serum albumin in Dulbecco's phosphate buffer. The tubes were vortexed and centrifuged for five minutes at 11000 g, whereafter the phosphate-32 activity in the supernatant was measured.

**SYNTHESIS OF PHOSPHATIC ACID IN GLYCEROL LABELLED CELLS**

Granulocytes from 70 ml of blood were suspended in 2 ml of HBSS containing 0.5 mM CaCl₂ and incubated for one hour at 37°C with 740 kBq [³H]glycerol (NET 022, New England Nuclear).

The cells were washed twice and resuspended in HBSS. Cells from different donors were combined immediately before the experiment.

The cell suspension, 15×10⁶ cells in 500 µl containing 5 µg/ml of cytochalasin B (Calbiochem) and the test compound as indicated were preincubated for 10 minutes at 37°C. Thereafter the cells were stimulated by addition of 0.1 or 1 µM fMLP. The reaction was terminated by addition of 1.875 ml chloroform/methanol (1:2, v/v) after 60 seconds. To obtain phase separation, 625 µl of chloroform and 625 µl of water were added. The lower phase containing the phospholipids was collected and evaporated.

For the phosphatidic acid determinations the phospholipids were dissolved in 30 µl chloroform/methanol/water (75:25:2, v/v). Exogenous phosphatidic acid, 20 µg, and phosphatidylinositol, 10 µg, were added as carriers making the total volume 50 µl. The phospholipids were separated by thin layer chromatography on Silicagel 60 plates (5715, E. Merck, Darmstadt, Germany) essentially as described previously. The mobile phase consisted of methyl acetate/propan-1-ol/chloroform/methanol/0.25 M KCl/acetic acid (25:25:25:12:9:0-062, v/v). After the chromatographic separation the phospholipid spots were stained with iodine vapour. The spots corresponding to phosphatidic acid, phosphatidylinositol, and phosphatidylcholine were scraped and counted in a scintillation counter.

**PHOSPHOLIPASE D ACTIVITY**

Radiolabelling of granulocytes with [³H]alkyl-lysophosphatidylincholine (octadecyl-lyso platelet activating factor, TRK.745, Amersham, Solna, Sweden), cell activation and analysis were performed essentially as described by Billah et al. Briefly, 20× 10⁶ cells/ml were suspended in 25 mM HEPES (pH 7.4) with 125 mM NaCl, 0.7 mM MgCl₂, 0.5 mM EGTA, 10 mM glucose, and 1 mg/ml albumin. Radiolabel, 185 kBq/ml was added and the suspension was incubated for 75 minutes at 37°C. After washing, the cells were resuspended to 5× 10⁶ cells/0.45 ml in HEPES buffer without albumin.

The cells were preincubated with cytochalasin B (5 µg/ml) and test compound for five minutes at 37°C, after which stimulus was added, either 0.1 µM fMLP, or 20 mM NaF plus 30 µM AlCl₃ or 0.1 µM phorbol-12-myristate-13-acetate (PMA, Sigma Chemical). The final sample volume was 500 µl, containing 5× 10⁶ cells. The reaction was terminated by addition of 1.875 ml chloroform/methanol/acetic acid (100:200:4, v/v). Chloroform and water (625 µl of each) were then added to obtain phase separation. The lower phase was collected and evaporated. For the determination of phosphatidic acid and diacylglycerol the phospholipids were dissolved in 30 µl chloroform/methanol/water (75:25:2, v/v). Phosphatidic acid, 20 µg, was added as carrier making the total volume 50 µl. The phospholipids were separated by thin layer chromatography as described by Billah et al. Their solvent systems I and III. After the chromatographic separation the phospholipid spots were stained with iodine vapour. The spots corresponding to phosphatidic acid and diacylglycerol were scraped and counted in a scintillation counter after addition of 0.25 ml methanol and 10 ml Instagel. Counts were corrected for quenching by sulphosalazine.

**STATISTICS**

Results are expressed as the mean (SD). Statistical significance was determined using Student’s t test. A p value of less than 0.05 was considered significant.

**Results**

**SYNTHESIS OF INOSITOL-1,4,5-TRISPHOSPHATE**

Initial experiments showed that the intracellular concentration of inositol-1,4,5-trisphosphate concentration was at a maximum about 30 seconds after activation with 0.1 µM fMLP. The radioactivity in the inositol-1,4,5-trisphosphate fraction was 79 counts/min before activation and 317, 374, and 175 counts/min 15, 30, and 60 seconds after activation in a representative experiment. A reaction time of 30 seconds was therefore chosen for studies of the effects of the drug.
Table 1: Inhibition of human granulocyte inositol-1,4,5-trisphosphate synthesis by sulphasalazine and its metabolites. The granulocytes were stimulated with 0.1 μM N-formyl-L-phenylalanine (fMLP) for 30 seconds. Results are given as mean (SD).

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Concentration (μM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphasalazine</td>
<td>100</td>
<td>45.6 (11.9) (n=8)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>28.5 (6.4) (n=2)</td>
</tr>
<tr>
<td>5-Aminosalicylic acid</td>
<td>100</td>
<td>13.8 (4.6) (n=4)</td>
</tr>
<tr>
<td>Sulphapyridine</td>
<td>100</td>
<td>7.5 (8.2) (n=4)</td>
</tr>
</tbody>
</table>

Table 1 shows that sulphasalazine (10 and 100 μM) inhibited the synthesis of inositol-1,4,5-trisphosphate. The metabolites sulphapyridine and 5-aminosalicylic acid at 100 μM inhibited the reaction significantly less than 10 μM of the mother molecule.

CYTOSOLIC CALCIUM

Figure 2 shows that a transient increase in cytosolic calcium occurred after stimulation with fMLP. The peak value was reached after about eight to 10 seconds (including time for stirring), and was followed by a less rapid decline. The increase in cytosolic calcium varied between cells from different donors and was 85 (25) nmol/l for 1 nM fMLP in the presence of extracellular calcium (12 subjects). In the absence of extracellular calcium it was 70 (17) nmol/l (six subjects) and 126 (69) nmol/l (nine subjects) respectively for 1 and 10 nM fMLP.

Table 2: Effect of sulphasalazine and sulphapyridine on the increase of cytosolic calcium in human granulocytes. The results are mean (SD) percentage inhibition of the peak value, which was reached about 10 seconds after addition of the stimulus.

<table>
<thead>
<tr>
<th>Stimulus*</th>
<th>Test compound</th>
<th>Concentration (μM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium with extracellular Ca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 nM fMLP</td>
<td>Sulphasalazine</td>
<td>5</td>
<td>34.2 (6.3) (n=6)</td>
</tr>
<tr>
<td></td>
<td>Sulphasalazine</td>
<td>10</td>
<td>61.1 (12.1) (n=5)</td>
</tr>
<tr>
<td></td>
<td>Sulphapyridine</td>
<td>10</td>
<td>12.4 (7.5) (n=4)</td>
</tr>
<tr>
<td>Medium with EGTA, without Ca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 nM fMLP</td>
<td>Sulphasalazine</td>
<td>5</td>
<td>47.8 (13.7) (n=4)</td>
</tr>
<tr>
<td></td>
<td>Sulphasalazine</td>
<td>10</td>
<td>67.7 (7.9) (n=5)</td>
</tr>
<tr>
<td>10 nM fMLP</td>
<td>Sulphasalazine</td>
<td>10</td>
<td>30.6 (7.4) (n=7)</td>
</tr>
<tr>
<td></td>
<td>Sulphapyridine</td>
<td>10</td>
<td>10.5 (6.0) (n=5)</td>
</tr>
</tbody>
</table>

* fMLP = N-formyl-L-phenylalanine.

GTTP INDUCED SUPEROXIDE PRODUCTION

Electropermeabilised granulocytes stimulated with GTP[S] (tetraphosphite, 50 μM) produced enough superoxide to reduce 8·1 nmol cytochrome c for each 50 000 cells (table 3). The production of superoxide after stimulation with 10 μM GTP[S] was slightly less, or 6·8 nmol for each 50 000 cells.

The superoxide production was inhibited in the presence of sulphasalazine (100 μM). The inhibition varied between blood donors and was more pronounced after stimulation with 10 μM GTP[S] (43–87% inhibition) than with 50 μM GTP[S] (23–39% inhibition). GTP[S] did not stimulate superoxide production in intact (not electropermeabilised) cells (not shown).

In the original method, 1 mM ATP was added to the reaction mixture. Exogenous ATP is, however, known to activate granulocytes leading to formation of inositol phosphates and an increase in Ca++ , and to prime granulocytes for enhanced superoxide production after fMLP stimulation. Favourably, in contrast to Nasmith et al24 and in agreement with Hartfield and Robinson,30 we found that the superoxide production did not require exogenously added ATP.

SYNTHESIS OF PHOSPHATIDIC ACID IN GLYCEROL LABELLED CELLS

The response to stimulation with fMLP varied considerably between cells from different donors. The relative increase of radioactivity in the phosphatic acid band 60 seconds after fMLP stimulation was 1·74 (0·22) fold (seven

Table 3: Effect of 100 μM sulphasalazine on superoxide production by electropermeabilised human granulocytes stimulated with guanosine 5'-(y-thio)triphosphate (GTTP[S]). The superoxide production is expressed in nanomol reduction of cytochrome c per sample containing 500 000 cells. Results are given as mean (SD) (n=4).

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Control</th>
<th>Sulphasalazine</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM GTTP[S]</td>
<td>8.1 (1.3)</td>
<td>5.6 (1.4)</td>
<td>31 (8)</td>
</tr>
<tr>
<td>50 μM GTTP[S]</td>
<td>8.1 (1.3)</td>
<td>5.6 (1.4)</td>
<td>31 (8)</td>
</tr>
</tbody>
</table>
Table 4 Inhibition of human granulocyte phosphatidic acid synthesis by sulphasalazine and metabolites. The glycerol
labelled granulocytes were stimulated with 0·1 µM N-
formyl-L-methionyl-L-leucyl-L-phenylalanine for 60 seconds.
The inhibition is expressed as mean (SD) percentage of the
phosphatidic acid synthesis in the absence of test compound.

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Concentration (µmol/l)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphasalazine</td>
<td>100</td>
<td>60·5 (18·6) (n=14)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>42·5 (27·6) (n=4)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>38·9 (19·0) (n=8)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>26·3 (8·4) (n=4)</td>
</tr>
<tr>
<td>5-Aminosalicylic acid</td>
<td>100</td>
<td>51·2 (15·3) (n=4)</td>
</tr>
<tr>
<td>Sulphapyridine</td>
<td>100</td>
<td>38·8 (18·2) (n=4)</td>
</tr>
</tbody>
</table>

Samples) for 1 µM, and 1·73 (0·42 fold (29 samples) for 0·1 µM fMLP.
Sulphasalazine (100 µmol/l) inhibited the increase in phosphatidic acid after stimulation with fMLP (1 µmol/l) by 54 (14)% (seven samples). At 0·1 µM fMLP, the inhibition in the presence of 100 µM sulphasalazine was 67 (19)%, and the inhibition was dose dependent with respect to sulphasalazine (table 4). The metabolites sulphapyridine and 5-aminosalicylic acid inhibited phosphatidic acid synthesis only about one tenth as potently as sulphasalazine.

SYNTHESIS OF PHOSPHATIC ACID AND DIACYLGLYCEROL IN PHOSPHATIDYLCHOLINE LABELLED CELLS

The phospholipase D mediated synthesis of phosphatidic acid after stimulation with 0·1 µM fMLP varied considerably between cells from different donors (fig 3A). Initial experiments showed that the maximum concentration of phosphatidic acid was obtained after 30–60 seconds, which agrees with earlier data. Table 5 shows that sulphasalazine (100 µmol/l) was a potent inhibitor of phosphatidic acid formation after 30 and 120 seconds, also 10 µM sulphasalazine inhibited the reaction significantly. Sulphapyridine (100 µmol/l) inhibited the reaction slightly whereas 5-aminosalicylic acid was inactive.

Phosphatidic acid is metabolised to diacylglycerol and the diacylglycerol synthesis consequently lagged behind the phosphatidic acid synthesis (fig 3B) in time, which agrees with earlier results. The synthesis of diacylglycerol was inhibited by sulphasalazine (table 5).

An accelerating increase in the concentration of phosphatidic acid after stimulation with 20 mM fluoride was seen for at least 10 minutes. Table 5 shows that 100 µM sulphasalazine inhibited the synthesis of phosphatidic acid by 56% after five minutes of stimulation.

Phosphatidic acid was produced in cells activated with PMA, essentially as described previously. No inhibitory effect of 100 µM sulphasalazine was found after five minutes (table 5).

Discussion

This study has shown that sulphasalazine inhibits the activation of human granulocytes through inhibition of the production of second messenger compounds derived from the GTP binding protein mediated activation of phospholipases C and D.

In the first set of experiments it was shown that sulphasalazine inhibited the phospholipase C dependent synthesis of inositol-1,4,5-trisphosphate from phosphatidylinositol-4,5-bisphosphate as well as the increase in cytosolic calcium in granulocytes. The latter inhibition may be secondary to the inhibition of inositol-1,4,5-trisphosphate synthesis, as cytosolic calcium is released by the action of inositol-1,4,5-trisphosphate. When a low concentration of fMLP (1 nmol/l) was used to stimulate the release of intracellular calcium, the inhibitory potency of sulphasalazine was increased (table 2). A similar finding was reported by Carlin et al, where it was found
that sulphasalazine inhibited granulocyte superoxide production better at lower fMLP concentrations. Kanerud et al. found, by using a single excitation wavelength, that 10 μM sulphasalazine inhibited the increase in cytosolic calcium in granulocytes stimulated with 100 nM fMLP.

The inhibitory effects of sulphasalazine showed that the drug affected the intracellular signalling system either at the level of plasma membrane receptor, the GTP binding protein coupled to the receptor, or the enzymatic activity of phospholipase C.

The first possibility (suggested by Stenson et al.) was addressed in experiments using electropermeabilised granulocytes activated with the GTP analogue GTP[S], thus bypassing the plasma membrane receptors. Sulphasalazine did inhibit the granulocyte activation with GTP[S], which revealed that the drug effect was not due to inhibition with the plasma membrane receptor for fMLP. Inhibition of the fMLP receptor as the major effect of sulphasalazine is unlikely, as earlier studies have shown that the drug inhibits cell activation by several receptor dependent stimuli other than fMLP, such as immune complexes and zymosan.

Phospholipase C is considered to be activated by the α subunit of the GTP binding protein. This protein is active when associated with a molecule of GTP, and deactivation is accomplished by hydrolysis of the GTP by an intrinsic GTPase activity. It is thus possible that sulphasalazine inhibits granulocyte activation by enhancing the hydrolysis of GTP. No effect of sulphasalazine on the GTPase activity was noted in the experiments designed to study this function, however.

The two products of phospholipase C mediated degradation of phosphatidylinositol-4,5-bisphosphate, inositol-1,4,5-trisphosphate and diacylglycerol, are considered to be the major intracellular signalling substances produced by the cells as a response to fMLP stimulation. In addition, the importance of phosphatidic acid for cell activation has attracted attention. It is likely that hydrolysis of phosphatidylcholine to phosphatidic acid is of physiological importance as a signal transduction pathway in the activation of, for example, granulocytes, which emphasises that the phosphatidic acid is more than a precursor, or a metabolite, of diacylglycerol.

The synthesis of phosphatidic acid by granulocytes labelled with glycerol was inhibited by sulphasalazine, thus confirming our earlier results using cells labelled with phosphate-32. As these earlier measurements, the recorded phosphatidic acid was mainly derived from diacylglycerol phosphorylated with phosphate-32 by the action of diacylglycerol kinase, the effect of sulphasalazine might have been due to inhibition of either the diacylglycerol kinase or to the synthesis of diacylglycerol.

The inhibition of the phosphatidic acid synthesis by sulphasalazine was similar in magnitude in cells with unspecifically (glycerol) labelled phospholipids and in cells specifically labelled in phosphatidylcholine. This similarity agrees with some studies which have shown that almost all the diacylglycerol formed during cell activation emanates from dephosphorylation of phosphatidic acid produced by phospholipase D mediated hydrolysis of phosphatidylcholine.

The effects of sulphasalazine on phospholipase D mediated degradation of phosphatidylcholine were studied by specific labelling of this phospholipid. Sulphasalazine inhibited the synthesis of phosphatidic acid and the subsequent formation of diacylglycerol in cells activated with fMLP. Fluoride induced activation was also inhibited, again demonstrating that the effect of sulphasalazine was not interaction with a plasma membrane receptor. Sulphasalazine did not inhibit PMA induced phospholipase D activity, which clearly shows that the drug did not inhibit phospholipase D, but interfered with an earlier step in the fMLP and fluoride induced activation of this enzyme.

The mechanism of hormone induced phosphatidylcholine hydrolysis are not known to the same extent as for phosphoinositide hydrolysis. Stimulation of granulocytes with fMLP or fluoride induces phospholipase D mediated phosphatidylcholine hydrolysis in addition to phospholipase C mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate. As cell stimulation with calcium ionophores and protein kinase C activating phorbol esters activates phospholipase D without phospholipase C involvement, it is possible that phospholipase D activation requires previous phospholipase C mediated hydrolysis of phosphatidylcholine, which is bypassed by hormone stimulation.

If so, the inhibition of sulphasalazine or the synthesis of phosphatidylcholine derived phosphatidic acid seen in this study is the consequence of a previous inhibition of phospholipase C. Alternatively, phospholipase D may not require previous phospholipase C activity but may be linked to a GTP binding protein, perhaps different from that linked to phospholipase C.

It can be concluded from the results of this study that sulphasalazine inhibited cellular activation by interaction with the synthesis of phosphoinositide derived second messenger compounds at the level of phospholipase C or its regulatory GTP binding protein. Sulphasalazine also inhibited the synthesis of the putative second messenger phosphatidic acid, which may be due to either the same mechanism, or by interaction with a phospholipase D regulating GTP binding protein. The metabolites of sulphasalazine, 5-aminoalicylic acid and sulphasalazine, were less potent inhibitors than the mother compound.


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18 Hurst N P, French J K, Gorisztchko L, Betts W H. Studies in parotid hyperfunction on the mechanism of inhibition of cholinergic tripeptide stimulated human neutrophil polymorphonuclear leucocyte superoxide production by chloroquine and hydroxychloro-


Sulphasalazine inhibition of human granulocyte activation by inhibition of second messenger compounds.

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