Biochemical Pharmacology, Vol. 35, No. 14, pp. 2443–2445, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

SHORT COMMUNICATION

Gentisic acid: an aspirin metabolite with multiple effects on human blood polymorphonuclear leukocytes

(*Received* 23 *December* 1985; *accepted* 20 *January* 1986)

Acetylsalicylic acid (aspirin) is commonly used in clinical practice as an anti-inflammatory agent and in the secondary prevention of myocardial infarction [1]. It blocks the conversion of arachidonic acid into cyclic endoperoxides [2]; this is the first step of production of prostaglandins, some of which have pro-inflammatory activity and/or promote platelet aggregation.

In several clinical trials on the prevention of myocardial infarction the aspirin dosage used was much higher than that necessary for completely inhibiting platelet aggregation [3].

Aspirin, which is still widely used at high doses in rheumatic disease, is metabolized to salicylic acid, which has a much longer half life and progressively accumulates [4]. Salicylic acid is eliminated from the body by a combination of processes including the formation of gentisic acid; while this is a first-order process, the formation of other metabolites, like salicyluric acid, is a capacity-limited process [5–7]. In rheumatic disease, and in all cases when aspirin is used at high doses, these kinetic characteristics might be responsible for an accumulation of gentisic acid in the body [4].

Recently it was suggested that drugs which reduce the migration and/or activation of leukocytes might be useful in reducing infarct size and in lowering the incidence of cardiac arrhythmias during infarction. The formation of aggregates of leukocytes in the vascular compartment with subsequent embolization in capillary networks has been proposed as a mechanism of tissue injury in several pathological states, e.g. in myocardial infarction [6].

We now report that gentisic acid *in vitro* is an inhibitor of human polymorphonuclear blood leukocyte aggregation and superoxide anion release after challenge with appropriate stimuli, while aspirin and sodium salicylate are both significantly less effective.

Our evidence suggests that gentisic acid might be directly involved in the broad therapeutic activity of its parent compound, aspirin.

We have studied the aggregatory response of PMN stimulated with arachidonic acid or compound A 23187, a calcium ionophore (Fig. 1). While aspirin and salicylate did not modify aggregation induced by either stimulus, gentisic acid produced concentration dependent inhibition. It was already apparent after 2 min preincubation with PMN and

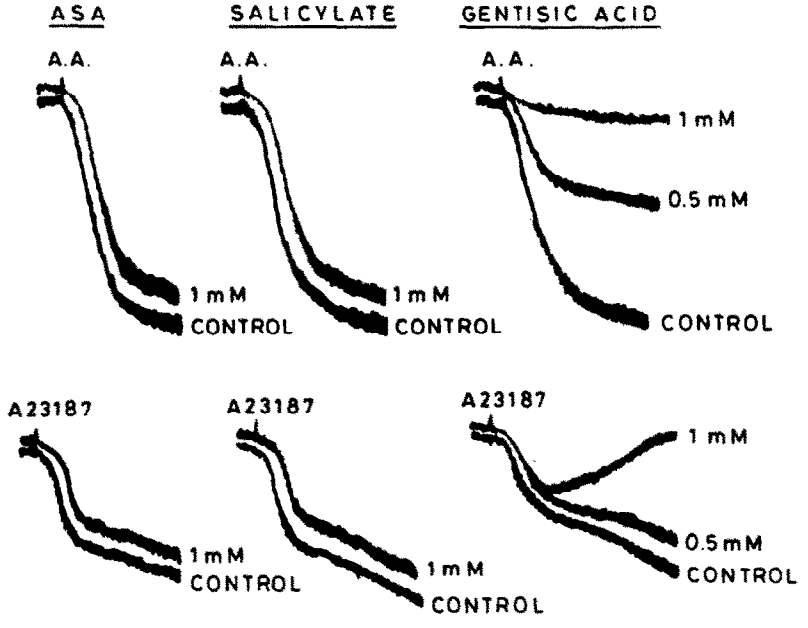

Fig. 1. Representative tracings of arachidonic acid (0.1 mM) or A-23187 (0.5 mM)-induced aggregation of PMN incubated for 2 min at 37° in the presence or absence of acetylsalicylic acid, sodium salicylate and gentisic acid. AA sodium salt (99% pure, Sigma Chem. Co., St. Louis, MO) was dissolved in 100 mM Na_2CO_3 and stored under nitrogen. The calcium ionophore A 23187 was dissolved in ethanol. PMN were obtained from citrated venous blood samples collected from healthy donors who had abstained from all medication for at least 10 days before blood donation; PMNs were isolated from the remaining blood by Dextran T 500 (Pharmacia Fine Chemicals, Uppsala, Sweden) sedimentation followed by Ficoll-Hypaque (Lymphoprep Nyegard, Oslo, Norway) density gradient centrifugation.

After washing, cells were suspended at the desired concentration in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution. Ca^{2+} and Mg^{2+} (0.6 and 0.8 mM, final concentrations) were added to cell preparations immediately before aggregation studies. The preparations routinely contained more than 97% PMN. The ratio of platelets to leukocytes in cell preparations was always less than 0.5:1 as determined by light microscopy. Cell viability was always more than 95% as measured by the Trypan blue exclusion test.

increased on prolonging the incubation period to 30 min. In the latter condition, the minimal concentration of gentisic acid required to suppress PMN aggregation completely was less than half that effective after 2 min. This is of interest since in subjects under chronic aspirin treatment, gentisic acid might interact with blood or extravascular leukocytes for long periods of time and may become effective even at low concentrations. The action of gentisic acid was not affected by previous incubation of PMN with a ten-times higher concentration of acetylsalicylic acid or sodium salicylate; this finding leads to the hypothesis that these compounds do not interact with the same PMN receptor.

In response to phorbol esters, like PMA (phorbol myristate acetate) or to arachidonic acid, PMN produce free radical species, particularly superoxide anion (O_2^-). Superoxide anion production was determined by measuring the SOD (superoxide dismutase)-inhibitable reduction of ferricytochrome C at 550 nm [9–11]; PMNs were preincubated for 5 min in the presence or absence of equimolar doses of aspirin, sodium salicylate and gentisic acid before challenge with PMA or arachidonic acid.

Gentisic acid had a significantly greater inhibitory effect than aspirin or salicylate on (O_2^-) production, when the cells were stimulated with arachidonic acid (Table 1). We also investigated changes in membrane fluidity, a major parameter subject to change during aggregation [12–14]. Since gentisic acid has some antiaggregating effect, we expected to see also a stabilizing effect on membrane fluidity.

Figure 2 shows that PMN membrane microviscosity (fluorescence polarization) changes after the addition of A 23187. Pretreating cells with gentisic acid caused significantly smaller changes in membrane microviscosity ($P < 0.01$). Pre-treatment of PMN with equimolar doses of aspirin or sodium salicylate did not affect A 23187-induced membrane microviscosity changes. This appears to confirm the protective effect of gentisic acid against the aggregation challenge also from the point of view of membrane microviscosity.

Table 2 shows the changes in membrane microviscosity with time; absolute values of fluidity increase were sig-

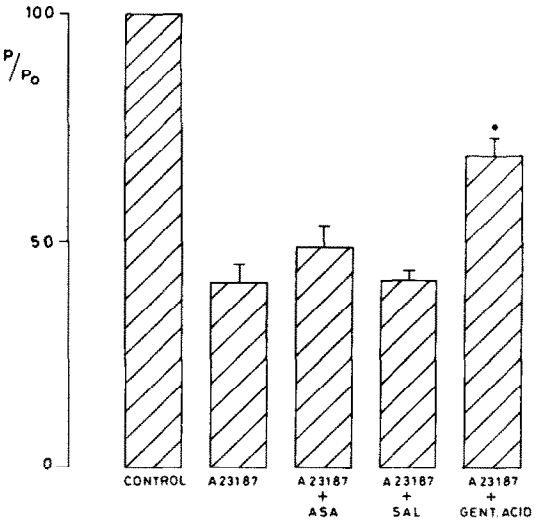

Fig. 2. Membrane fluidity changes were determined according to Shinitzky and Barenholz using an Elscint MV-1 microviscosimeter (Elscint, Haifa). Human PMN were incubated for 30 min at 4° with PBS containing 2 μM diphenylhexatriene, all-trans (DPH). After this time, for determination of changes in membrane microviscosity, 3 ml of the cell suspension were put into the cuvette and the basal fluorescence polarization value was recorded. The aggregating stimulus was then added to the cuvette and the fluorescence polarization value was recorded until no further change was detectable (usually 3 min). Membrane microviscosity was calculated according to the formula

$$n = \frac{2P}{0.46\text{-}P}$$

Possible optical interference by ASA, salicylate and gentisic acid was minimized by carefully washing the cells immediately before use.

Table 1. Production of (O_2^-) by polymorphonuclear leukocytes stimulated with 0.2 mM arachidonic acid in presence of various drugs

	(O_2^-) Released (nmol/3 hr/10^6 cells)	% Inhibition
Control	3.5 ± 0.11	—
ASA 1 mM	2.0 ± 0.05*	43
Salicylate 1 mM	2.1 ± 0.07*	40
Gentisic acid 1 mM	0.09 ± 0.01**	97

Each value is the mean ± S.E. of 5 different determinations.
* $P < 0.05$ and ** $P < 0.01$ in respect of control values.

Superoxide anion production by human blood polymorphonuclear leukocytes was determined by measuring the superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C to ferrocytochrome C at 550 nm. Briefly, cells suspended at a concentration of 5×10^6 cells/ml in Hank's balanced salt solution containing 0.1 mM ferricytochrome C were preincubated at room temperature for 5 min in the presence or absence of the test reagents (aspirin, sodium salicylate, gentisic acid). The reactions were started by addition of the specific stimulus in a final volume of 0.2 ml and changes in optical absorbance at 550 nm was monitored using a Multiscan Titertek spectrophotometer (LKB). The amount of O_2^- produced was calculated from the difference in absorbance between samples of cells that received SOD before activation by the stimulus and those receiving SOD after activation. The difference was divided by the extinction coefficient for the change between ferricytochrome C and ferrocytochrome C to determine nmoles of O_2^- produced per minute per given quantity of cells. The data are expressed as mean values for triplicate samples ± S.D.

Table 2. Time-course of fluorescence polarization variation after the addition of 0.5 mM A 23187

Time (min)	Control	ASA	Salicylate	Gentisic acid
0	0.230 ± 0.002	0.218 ± 0.004	0.222 ± 0.005	0.192 ± 0.004
0.5	0.213 ± 0.005	0.203 ± 0.009	0.191 ± 0.008	0.183 ± 0.004*
1	0.096 ± 0.010	0.106 ± 0.010	0.090 ± 0.003	0.147 ± 0.014**
2	0.091 ± 0.010	0.103 ± 0.011	0.092 ± 0.004	0.130 ± 0.005**
3	0.095 ± 0.009	0.106 ± 0.010	0.095 ± 0.003	0.133 ± 0.006**

Each value is the mean ± S.E. of at least 5 different determinations.
* $P < 0.05$ and ** $P < 0.01$ versus control values.
The variation is shown of fluorescence polarization in PMN pretreated with the same concentration (2 mM) of aspirin (ASA), salicylate and gentisic acid, as detailed in the legend to Fig. 2.

nificantly different ($P < 0.01$) at all times except 0 (before addition of A 23187), indicating that gentisic acid had no direct effect on cellular membranes. Therefore, an effect of cell membrane rigidification, leading to decreased susceptibility to the aggregating challenge, can be excluded.

Aggregation and superoxide production in human granulocytes should probably be considered as signals of an activated cellular state, temporally but not necessarily causally related, as recently shown by Whitin and Cohen [15]. The effect of gentisic acid on PMN membrane fluidity, on the other hand, might be closely related to the aggregation inhibition, since a large increase in membrane rigidity might counteract the effect of the aggregating agents on PMN.

We are currently pursuing a better understanding of the clinical relevance of our findings. Plasma levels of gentisic acid after administration of 1.5 g of aspirin or sodium salicylate, although relatively low (about 0.5 mg/l) could nevertheless be measured for more than 15 hr [6]. On the other hand, a dose-dependent percentage increase of gentisic acid formation in man was reported after aspirin administration; gentisic acid accounted for up to 3.3% of aspirin doses during a schedule of 4 g daily [16]. Especially in patients undergoing high-dose aspirin therapy, the levels of gentisic acid in plasma or in extracellular fluids might be sufficient to exert profound metabolic and functional effects on leukocytes.

Acknowledgements—Judith Baggott, Ivana Garimoldi, Vincenzo and Felice de Ceglie and the staff of G. A. Pfeiffer Memorial Library helped prepare the manuscript. This work was supported by the Italian National Research Council (CNR), Progetto Finalizzato "Medicina Preventiva", Sottoprogetto "Malattie Degenerative" Contract No. 85.00501.56.

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