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INHIBITION BY N-ACETYL NEURAMINIC ACID OF PLATELET THROMBOGENESIS INDUCED BY LASER INJURY TO RAT AND HAMSTER VENULES

GUSTAV V.R. BORN & IREN B. KOVÁCS[1]

Department of Pharmacology, University of Cambridge, Cambridge, CB2 2QD

1 In rats and hamsters under barbiturate anaesthesia, laser radiation to venules about 50 μm in diameter in mesoappendix and cheek pouch respectively caused the formation of platelet thrombi which occluded the vessels in about 9 min.

2 This occlusion time was significantly prolonged by the intravenous injection of N-acetyl neuraminic acid (NANA) but not by D-glucuronic acid or β-methoxyneuraminic acid, in doses which had no effect on blood pH or on the condition of the animals.

3 The results confirm the anti-thrombotic effect of NANA previously demonstrated with another technique.

Introduction

In cheek pouch venules of anaesthetized hamsters, mural thrombi of platelets are formed when adenosine diphosphate (ADP) is applied micro-iontophoretically close to the outside of the vessels without any demonstrable damage to their wall (Begent & Born, 1970; Begent, Born & Sharp, 1972). One way of quantifying this effect is to measure the time from the application of the iontophoretic current to the first microscopic appearance of thrombotic material opposite the tip of the micropipette (Begent *et al.*, 1972). Under these conditions, a single intravenous injection of neuraminidase (EC3.2.1.18 from *Vibrio cholerae*) 0.09 iu in 1.5 min, greatly increased this thrombus delay time, indicative of an inhibitory effect on the intravascular adhesion and aggregation of platelets (Atherton & Born, 1973). Another effect of the injected neuraminidase was to raise the concentration of free sialic acid in the animals' plasma to about 60 μg/per ml. When, instead of the enzyme, enough (2000 μg) N-acetyl neuraminic acid (NANA) was injected intravenously to produce a similar rise in plasma sialic acid concentration, platelet thrombus formation was again inhibited, if anything more strongly (Atherton & Born, 1973). Neither the neuraminidase nor the NANA affected the concentrations of circulating platelets and neither agent produced any deleterious effects in the animals.

These observations suggested that the search for an agent capable of inhibiting platelet thrombosis should be extended to naturally occurring sialic acids and synthetic analogues. With this in mind, more experiments have now been done which confirm and extend the original observations with a different experimental technique in another species of animal.

[1] Present address: Otto Korvin Hospital, Budapest, Hungary.

Methods

Animal preparations

Rats, Wistar males, weighing 130 to 150 g were anaesthetized with pentobarbitone sodium (6 mg/100 g body weight injected subcutaneously) and the mesoappendix was prepared for microscopic observation of its venule (Zweifach & Metz, 1965).

Golden hamsters of either sex weighing 80 to 100 g were anaesthetized similarly and a layer of the cheeck pouch was prepared for microscopic observation of venules and calculation of the volume of platelet thrombi (Begent & Born, 1970). Injections were made over periods of 1.5 min through the femoral vein in rats and the jugular vein in hamsters.

Platelet thromobogenesis by laser damage (Kovács, Csalay & Görög, 1973; Kovács, Tigyi-Sebes, Trombitas & Görög, 1975)

The beam from a He–Ne laser (Spectraphysics, 15 mW) was passed through the microscope (Leitz Orth-

olux) and aimed through the objective at target venules, 40 to 60 μm in diameter. At the target the diameter of the laser beam was 15 μm. Before the first irradiation, Evans blue (T 1824) at a concentration of 10 mg/ml of 0.9% w/v NaCl solution (saline) containing 3% bovine albumin was injected intravenously at a dose of 20 mg/kg body weight. The circulating dye served as energy-absorbing material to ensure that the laser radiation would damage the inside of the vessel. Platelet thrombus formation which followed laser irradiation was observed microscopically both directly and via a television image enlarged on a video monitor. Thrombus growth was quantified in two ways. In one, target venules were laser-irradiated for 5 s every minute and the time was determined from the first irradiation until the thrombus completely occluded the flow of blood, the 'occlusion time'. In the other, target venules were irradiated for 10 s only once; the growth of the resulting thrombus was recorded on video tape from which the thrombus growth rate was estimated (Begent & Born, 1970).

N-acetyl neuraminic acid from *E. coli*, β-methoxy neuraminic acid, D-glucuronic acid and bovine albumin were from the Sigma Chemical Company. Each compound was dissolved in saline at appropriate concentrations (see Results) immediately before use.

Results

In rats, platelet thrombi resulting from laser damage to mesoappendix venules always stopped the blood flow through them with a mean occlusion time of just over 9 min (Table 1). Intravenous injections of NANA prolonged the occlusion time increasingly with increasing doses; with the highest dose, 7 out of 9 venules were not occluded at all by the time observation was discontinued after 15 min (Table 1). When looked at again after about 20 min some of these venules contained small mural thrombi.

These effects, produced by NANA injected as the free acid at pH 2.3, caused no observable change in the pH of blood samples taken after 2 min, in the blood flow through the venules or in the concentration of circulating platelets. Nevertheless, it was

Table 1 Effects of equimolar N-acetyl neuraminic acid (NANA), glucuronic acid or neuraminic acid β-methylglycoside on laser-induced thrombus growth in rat mesoappendix venules

Agent	*Dose injected (μmol/kg i.v.)*	*No. of venules irradiated*	*No. of venules open after 15 min*	*Occlusion time (min) (mean ± s.e. mean)*†
None	Nil	23	0	9.2 ± 0.4
NANA	16	6	3	12.5 ± 1.2(1)
NANA	24	15	5	13.4 ± 0.5(2)
NANA	32	9	7	14.1
Glucuronic acid	32	10	0	9.5 ± 0.8
Neuraminic acid β-methylglycoside	32	9	0	9.7 ± 0.7

† Calculated from veins occluded by 15 min
(1) $P < 0.01$; (2) $P < 0.001$.

Table 2 Effect of N-acetyl neuraminic acid (NANA) on laser-induced thrombus growth in hamster cheek pouch venules

Agent	*Dose injected (μmol/kg i.v.)*	*No. of venules irradiated*	*No. of venules open after 15 min*	*Occlusion time (min) (mean ± s.e. mean)*†
Control	—	10	0	10.8 ± 0.6
NANA	24	4	1	13.5 ± 0.7(1)
NANA	32	22	19	4.7 ± 0.2(2)

† See Table 1
(1) $P < 0.02$; (2) $P < 0.001$.

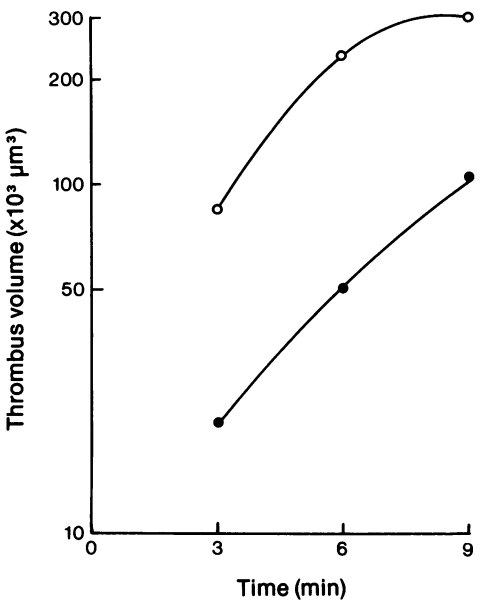

Figure 1 Volume of platelet thrombus, calculated according to Begent & Born (1970), growing in hamster cheek pouch venules, about 50 μm in diameter, after laser irradiation in the absence (○) and presence (●) of N-acetyl neuraminic acid injected intravenously at zero time in a dose of 32 μmol/kg body weight. The differences were statistically significant after 3 ($P < 0.002$) and 6 ($P < 0.01$) min.

desirable to determine whether intravenous injections of another organic acid similar to NANA and with a similar pK_a inhibited platelet thrombogenesis as did NANA. D-Glucuronic acid injected at the same molar concentration as the highest dose of NANA had no significant effect on the occlusion time, nor had a NANA analogue, β-methoxy neuraminic acid, injected at the same concentration (Table 1).

In hamsters, the effect of NANA on platelet thrombi produced by laser injury to venules in cheek pouch preparations was similar to its effect in rats (Table 2). The growth rate of the thrombi in this preparation was significantly diminished by NANA (Figure 1).

Injections of NANA had no observable deleterious effects on the animals of either species.

Discussion

The results show that NANA, injected intravenously in concentrations that had no adverse effects on anaesthetized rats or hamsters, strongly inhibited the formation of adherent platelet thrombi in venules damaged by laser irradiation. This confirms previous observations (Atherton & Born, 1973) in another species and with a different technique for producing platelet thrombi. To exclude the possibility that the inhibition was due merely to the acidity of the NANA, D-glucuronic acid which has a similar pKa value was injected at the same concentration as NANA without producing its inhibitory effect. β-Methoxy neuraminic acid was also inactive, suggesting that the acetyl substituent and/or the free hydroxyl on the C-2 atom were essential for inhibitory activity.

The mechanism by which laser radiation induces platelet thrombi to form in a vessel is not exactly known. However, it is reasonable to assume that the intense radiant energy damages the endothelial layer so that circulating platelets are able to collide with exposed collagen and to adhere to it. If it is the reaction between platelets and collagen that is inhibited by NANA, it should have similar activity in another system in which the interaction between collagen and platelets produces a quantifiable abnormality. Emboli of platelet thrombi can be produced in mice by the intravenous injection of collagen (Nishizawa, Wynalda, Suydam, Sawa & Schultz, 1972). Male mice weighing around 20 g were injected intravenously with a suspension of collagen prepared from cat Achilles tendons (Hovig, 1963). In normal mice, 0.4 ml of this collagen suspension administered intravenously produced a survival rate of about 20%; 10 out of 12 of these control mice died of respiratory failure in 2.48 ± 0.11 min (mean ± s.e. mean). When NANA at a concentration of 20 mg/kg was injected intravenously 30–60 s before the collagen, 10 out of 12 mice survived for observation periods of at least 5 h. These observations support the conclusion that the intravascular formation of platelet thrombi, at least as induced through their reaction with collagen, can be inhibited by N-acetyl neuraminic acid in concentrations that have no apparent deleterious effects on the animals. It may indeed be useful, therefore, to explore further the activity of naturally-occurring sialic acids and their synthetic analogues as antithrombotic agents.

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