Sex Related Differences in Platelet Function: The Effect of Aspirin

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There is evidence from clinical studies and animal experiments that aspirin has a greater antithrombotic activity in males compared to females. We investigated platelet function in vitro and in vivo in rabbits before and after the administration of a dose of aspirin (5 mg/kg) which inhibited collagen stimulated thromboxane B₂ generation. Infusion of collagen into untreated animals resulted in a 38 ± 4% (m ± SE, n = 13) decrease in platelet count (assessed by whole blood radioactivity) in the male animals, and a 27 ± 3% (m ± SE, n = 13) in the female animals. Pretreatment with aspirin resulted in a significant inhibitory effect in the male but not the female animals (p < 0.05). The male animals had significantly greater thromboxane B₂ generation in vivo than did the female animals following an equal dose of collagen (males, 2.64 ± 0.7 ng/ml thromboxane B₂, n = 14; females, 1.67 ± 0.4 ng/ml thromboxane B₂, n = 15, p < 0.05). In contrast no sex related difference in the inhibitory effect of aspirin on maximal collagen induced aggregation was found when platelets were studied in vitro. The greater reactivity of male patients in vivo may be accounted for by the observed increase in thromboxane B₂ generation. This might also explain the greater thrombotic tendency of males, and the observed difference in the antithrombotic effect of aspirin in males and females.

ASPIRIN irreversibly acetylates the platelet enzyme cyclooxygenase resulting in inhibition of synthesis of the platelet aggregating substance, thromboxane A₂. The antithrombotic activity of aspirin (ASA) has been evaluated in a number of clinical trials and reported to be effective in preventing arterial venous shunt thrombosis, stroke and death in patients with transient ischemic attacks, and in preventing postoperative thrombi after hip surgery. In several studies, the beneficial effects of aspirin were limited to males. We have previously reported that aspirin significantly decreased the size of vessel injury induced venous thrombi in male but not in female rabbits. In this report we describe investigations comparing the effects of aspirin in male and female rabbits on in vivo and in vitro rabbit platelet aggregation and thromboxane B₂ release.

MATERIALS AND METHODS

Preparation of Aspirin Solution

Aspirin (Sigma Chemicals, St. Louis, Mo.) was dissolved in equimolar sodium carbonate buffer solution (pH 7.0), and frozen in aliquots prior to use. A stock, 100 mg/ml, solution of aspirin was made by adding 10 g aspirin to 80 ml distilled water. Under constant agitation and while monitoring the pH, aliquots of a solution of 5 g sodium carbonate in 20 ml of water were slowly added to the aspirin so that the pH did not rise above 7.0. Less than 3% of the aspirin had hydrolyzed to sodium salicylate.

Animal Studies

New Zealand white rabbits, weight 2-3 kgs, were used in all experiments. The animals were anesthesiazed with 30 mg/kg sodium pentobarbital (MTC Pharmaceuticals, Hamilton, Ontario) administered intravenously through a marginal ear vein.

Inhibition of Thromboxane B₂ Generation by Aspirin

Male and female rabbits were infused with 1, 3, 5, 10 and 20 mg/kg of aspirin or sodium salicylate and 30 min later 5 ml of whole blood collected from a marginal ear vein into sodium citrate (9.0.9, v:v) containing 50 ul of collagen per ml. The collagen (final concentration, 0.25%, pH 2.8), was produced from Achilles tendon collagen, (Sigma Chemicals), using the technique previously described. The blood was gently mixed for 30 sec and the platelet poor plasma (PPP), separated by centrifugation at 13,000 g for 10 min. The amount of thromboxane B₂ in the PPP was measured using a radioimmunoassay.

Ex Vivo Platelet Aggregation Studies

Animals were treated with either aspirin (5 mg/kg) or sodium salicylate (5 mg/kg) 30 min prior to venesection. Whole blood was collected through a carotid cannula from male and female animals into 3.8 sodium citrate (9.0.9, v:v); the final citrate concentration was kept constant by adjusting the proportions according to the hematocrit. Platelet rich plasma (PRP) was obtained by differential centrifugation (260 g x 10 minutes) and the final platelet count adjusted to 200,000 per ul with platelet poor plasma, (PPP). Platelet aggregation was initiated with bovine acid soluble collagen, prepared as described. A single batch of collagen was used for all experiments described in this report. All aggregations were performed at 37°C in the same aggregometer, using 1 ml siliconized cuvettes and rotating bars. The recorder was set at 0% and 100% using PRP, PPP.

In Vivo Platelet Aggregation Studies

The details and validation of this method have previously been described. One rabbit served as a platelet donor for a recipient of the same sex. The donor rabbits were exsanguinated through a carotid cannula and the platelet rich plasma prepared as described previously. A platelet pellet was obtained by centrifugation for 10 min at 2300 g. The platelets were washed twice with calcium and albumin free Tyrode's solution, (pH 6.2), and incubated with 50 u Ci of ⁵¹sodium chromate, (⁵¹Cr.), (New England Nuclear, Boston, Mass.) for 30 min at 22°C. The platelets were washed twice with calcium

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free Tyrodes, resuspended in Tyrode's albumin, (pH 7.3) and infused into the recipient rabbits. The animals were then given aspirin (5 mg/kg) or saline (0.9%) by intravenous infusion. Thirty min later, 50 ul/kg of acid soluble bovine collagen was infused through a carotid cannula. One ml of whole blood was drawn from the rabbits via a jugular cannula at 30, 60, 90, 120 and 240 sec after infusion of the collagen, and the platelet count and whole blood radioactivity of these specimens measured.

**In Vivo Thromboxane B<sub>2</sub> Release**

Male and female rabbits were given 5 mg/kg of aspirin or sodium salicylate through a marginal ear vein and 30 min later 50 ul/kg of collagen infused intravenously. Whole blood for thromboxane B<sub>2</sub> measurement was collected before and 90 sec after collagen infusion into a chilled mixture of sodium citrate and indomethacin (5 mg/kg final concentration). The whole blood was centrifuged at 3000 g for 10 min and the thromboxane B<sub>2</sub> in the supernatant quantitated.

**Radioimmunoassay for Thromboxane B<sub>2</sub>**

Thromboxane B<sub>2</sub> was measured using modifications of a previously reported radioimmunoassay. Varying concentrations of standard thromboxane B<sub>2</sub> or test plasma were incubated with known concentrations of 3H-thromboxane B<sub>2</sub> (New England Nuclear, Boston, Mass., Lot 1118-255) plus antithromboxane B<sub>2</sub> antiserum, (a gift from Dr. J. W. D. McDonald). Human gammaglobulin (Cohn Fraction II: Sigma Chemicals, St. Louis, Mo.) was added to facilitate the separation of the bound and unbound fraction which was accomplished by the addition of saturated ammonium sulphate.

**RESULTS**

**The Effect of Aspirin on Inhibition of Thromboxane B<sub>2</sub> Production**

The in vitro collagen induced thromboxane B<sub>2</sub> biosynthesis of untreated animals, (two male, two female) was 332.5 ± 81.5 ng/ml (m ± SE) thromboxane B<sub>2</sub> and the thromboxane B<sub>2</sub> generated by the animals pretreated with 1, 5, or 20 mg/kg of sodium salicylate was 668.85 ± 62.6; 368.86 ± 56.4; 526.27 ± 172.9, (m ± SE, n = 2 male and 2 female animals for each dose). Pretreatment of the animals with doses of aspirin as low as 1 mg/kg caused significant inhibition of collagen induced thromboxane B<sub>2</sub> biosynthesis in vitro and pretreatment with 5 mg/kg resulted in >99% inhibition of TXB<sub>2</sub> generation. This dose of aspirin was used in all subsequent studies. The amount of collagen induced thromboxane B<sub>2</sub> generation (m ± SE, ng/ml) for two male and two female rabbits for each dose of aspirin was 1 mg/kg, 29.9 ± 13.75; 2 mg/kg, 3.3 ± 1.978; 5 mg/kg, 0.7 ± 0.600; 10 mg/kg, 0.1 ± 0.000; 20 mg/kg, 0.117 ± 0.018.

**Ex Vivo and In Vivo Platelet Aggregation**

The results of the ex vivo platelet aggregations performed on platelets obtained from rabbits pretreated with 5 mg/kg of aspirin or sodium salicylate are shown in Fig. 1. Platelet aggregation in the aspirin treated animals was compared with salicylate treated animals, (expressed as 100%) using a series of collagen dilutions. There was no difference between the maximal ex vivo platelet aggregation of male of female sodium salicylate treated animals over a wide range of collagen concentrations, (for each group n = 12). The degree of inhibition was inversely proportional to collagen concentration (Fig. 1).

The intravenous infusion of collagen caused a drop in the whole blood radioactivity which reached a nadir 90 sec after infusion (see Fig. 2). The drop in the whole blood radioactivity which was used to assess changes in platelet count was 38 ± 4%, (m ± SE, n = 13), and in the male animals 27 ± 3% (m ± SE, n = 13), in the female animals. Pretreatment with aspirin inhibited the fall in platelet count in the male, but not the female animals (p < 0.05). The post aspirin change in radioactivity in the male animals was 27 ± 3%, n = 15 and in the females it was 26 ± 2%, n = 14.
In Vivo Thromboxane B₂ Release

Prior to infusion of collagen, the level of plasma thromboxane B₂ was below the limits of detection of the radioimmunoassay (0.1 ng/ml). Ninety sec after infusion of the collagen (at the platelet nadir) there were significantly higher levels of thromboxane B₂ in plasma of the male animals compared to the female animals (males, $2.64 \pm 0.7$ ng/ml TxB₂; $n = 14$; females, $1.67 \pm 0.4$ ng/ml TxB₂; $n = 15$; $p < 0.05$, unpaired t-test).

DISCUSSION

The antiplatelet activity of aspirin is mediated by the acetylation of the enzyme cyclooxygenase which in turn prevents the synthesis of the platelet aggregatory substance, thromboxane A₂.¹ ² The results of clinical trials and one experimental animal study indicate that aspirin had a greater antithrombotic effect in males than in females.³ ⁴ ⁶ In this study we demonstrated that platelets in male animals have greater in vivo reactivity to collagen than platelets in female animals and that the increased reactivity is associated with and probably due to greater thromboxane generation in the male compared to the female animals. This conclusion is based on the following observations: 1) the infusion of collagen resulted in a greater fall in whole blood radioactivity (radiolabelled platelets) in male animals than in female animals 2) pretreatment of the animals with a dose of aspirin sufficient to inhibit thromboxane generation had a significant protective effect in the male but not in the female animals 3) the infusion of collagen resulted in significantly higher plasma levels of thromboxane B₂ in the male compared to the female animals. The results of the in vivo platelet aggregation studies also suggest that collagen induced thromboxane A₂ independent aggregation is similar in each sex.

In contrast to the results of the in vivo experiments, we were unable to demonstrate a sex related difference in in vitro platelet function even following correction for the effects of citrate on the hematocrit. The reason for the in vitro/in vivo difference is unknown.

Given the limitations of extrapolating results of animal studies to man, the greater sensitivity of male rabbit platelets to in vivo collagen induced aggregation and the associated increase in thromboxane B₂ generation could explain the greater thrombotic tendency reported for males and their greater response to aspirin.

REFERENCES
