Sex Difference in the Effect of Aspirin on Intracellular Ca^{2+} Mobilization and Thromboxane A_2 Production in Rat Platelets

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Abstract—The intracellular Ca^{2+} mobilization in thrombin-stimulated platelets was greater in male rats than in female rats. Thromboxane (TX) B_2 production in male platelets was greater than that in female platelets. Aspirin suppressed Ca^{2+} mobilization in rat platelets, but the inhibitory effect of aspirin was more efficient in males than that in females. Aspirin inhibited TXB_2 production, and this inhibitory effect of aspirin was stronger in male platelets than in female platelets. Castration decreased Ca^{2+} mobilization and TXB_2 production and weakened the effect of aspirin on them. It is suggested that the sex difference in the antiplatelet effect of aspirin results from the difference in the inhibition of Ca^{2+} mobilization via the inhibition of TXA_2 production in thrombin-stimulated rat platelets.

It has been demonstrated that men suffered from thrombosis more frequently than women (1). Platelets play an important role in the formation of thrombosis and the regulation of hemostasis. Aspirin suppresses platelet functions and has been accepted as a useful drug for antithrombosis (2). However, it was reported that there was a sex difference in the antithrombotic effect of aspirin (3). Aspirin inhibits the production of prostanoids, and it inhibits more strongly TXA_2 production in platelets than prostacyclin production in vessel wall (4). TXA_2 induces platelet aggregation and mediates platelet activation.

Ca^{2+} is an important regulator of cellular functions. Various stimulants induce Ca^{2+} influx and/or intracellular Ca^{2+} discharge and increase the concentration of cytoplasmic Ca^{2+} . It is suggested that Ca^{2+} serves as one of the second messengers of platelet activation. Aspirin and some other inhibitors of TXA_2 synthesis were reported to inhibit intracellular Ca^{2+} mobilization in platelets (5, 6). It was shown that TXA_2 increased the concentration of intracellular Ca^{2+} in human platelets (7).

In this work, we investigated the sex

differences in the inhibitory effects of aspirin on Ca^{2+} mobilization and TXB_2 production in rat platelets. In addition, we studied the influence of gonadectomy on the effects of aspirin on these items.

Materials and Methods

Materials: Thrombin (human plasma thrombin) was obtained from Midori Cross, aspirin from Sanko Seiyaku Co., quin 2-AM (2-[2-amino-5-methylphenoxy)methyl]-6-methoxy-8-aminoquinoline-N,N,N',N'-tetraacetic acid, tetra acetoxymethyl ester) and quin 2 from Dotite and [^{125}I] TXB_2 kit from Amersham. All reagents not specified above were of analytic grade.

Rats: Wistar strain rats of both sexes, aged 10 weeks, were used. Gonadectomy was performed as described in the previous report (8).

Drugs: Aspirin was suspended in 0.1% carboxymethylcellulose sodium salt solution for p.o. administration. The doses of aspirin were 5, 10 and 20 mg/kg.

Preparation of platelets: Blood was obtained from rat abdominal aorta at one hour after the administration of aspirin under ether anesthesia and was collected into a siliconized

tube containing 3.13% sodium citrate (9:1). Platelet-rich plasma was obtained by centrifugation at $170\times g$ for 10 min. Gel-filtered platelets (GFP) were prepared by using a Sepharose 2B column according to the method of Timmons and Hawiger (9).

Platelet count: Platelets were counted by the method of Brecher and Cronkite (10), and then GFP was diluted with the elution buffer to 1.0×10^8 platelets/ml.

Platelet Ca^{2+} mobilization: The GFP was incubated with $10\text{ }\mu\text{M}$ quin 2-AM at 37°C for 30 min. Then, the mixture was applied to a Sepharose 2B column by the method of Rink et al. (11). The external Ca^{2+} concentration was adjusted to 1 mM with CaCl_2 solution or to 0 mM with 2 mM EGTA. The platelets were maintained at 37°C , stimulated by 1.0 U/ml thrombin, and stirred magnetically throughout the measurement. The measurement of intracellular Ca^{2+} was performed principally by the method of Rink et al. (11). The concentration of the intracellular Ca^{2+} was measured from quin 2 fluorescence with a Hitachi fluorescence spectrophotometer 650–60. The excitation and the emission wavelengths were 339 and 492 nm, respectively.

Platelet TXB_2 production: After the measurement of intracellular Ca^{2+} mobilization, the reaction mixture was adjusted to pH 3.0 by the addition of 1 N HCl and extracted by ethyl acetate. The ethyl acetate layer was dried under nitrogen and kept at -40°C until the amount of TXB_2 was determined by radioimmunoassay using a [^{125}I] TXB_2 kit.

Results

Effect of aspirin on intracellular Ca^{2+} mobilization and TXB_2 production in thrombin-stimulated platelets: Figure 1 shows the effect of aspirin on Ca^{2+} mobilization in thrombin-stimulated platelets. Ca^{2+} mobilization in male platelets was greater than that in female platelets. Aspirin suppressed Ca^{2+} mobilization in rat platelets. However, the effect of aspirin was more efficient in male platelets (inhibitory rate at the dose of 10 mg/kg: 38.4% in males and 24.3% in females; at 20 mg/kg: 50.2% in males and 30.3% in females). In the absence of external Ca^{2+} ,

Fig. 1. Effect of aspirin on intracellular Ca^{2+} mobilization in rat platelets. The values are the mean $\pm$ S.E. ($n=7$). $\square$: Male, ▨ : Female. Platelets were stimulated by thrombin (1 U/ml). *1: Significant difference from the control value of each sex ($P<0.05$). *2: Significant difference from the value of control male ($P<0.05$).

Fig. 2. Effect of aspirin on TXB_2 production in rat platelets. The values are the mean $\pm$ S.E. ($n=7$). $\square$: Male, ▨ : Female. Platelets were stimulated by thrombin (1 U/ml). *1: Significant difference from the control value of each sex ($P<0.05$). *2: Significant difference from the value of control male ($P<0.05$).

Ca^{2+} mobilization significantly decreased, and the effect of aspirin on Ca^{2+} mobilization was suppressed.

Figure 2 shows the effect of aspirin on TXB_2 production in thrombin-stimulated platelets. TXB_2 production in male platelets was greater than that in female platelets. Aspirin inhibited TXB_2 production in rat platelets. Aspirin at a dose of 10 mg/kg inhibited TXB_2 production in male platelets, and at a dose of 20 mg/kg, significantly blocked it in both sexes. Moreover, aspirin

at a dose of 50 mg/kg completely inhibited TXB₂ production (data not shown).

Influence of gonadectomy on intracellular Ca²⁺ mobilization: Table 1 shows the influence of gonadectomy on the inhibitory effect of aspirin on Ca²⁺ mobilization in thrombin-stimulated platelets. In the control group, castration decreased Ca²⁺ mobilization in male platelets. However, ovariectomy did not influence Ca²⁺ mobilization.

Aspirin blocked Ca²⁺ mobilization in castrated male platelets, but the inhibitory effect of aspirin was weaker than that in intact male platelets. Aspirin suppressed Ca²⁺ mobilization in the platelets of ovariectomized rats in comparison with intact female platelets.

Influence of gonadectomy on TXB₂ production: Table 2 shows the influence of gonadectomy on the inhibitory effect of aspirin on TXB₂ production in thrombin-

Table 1. Influence of gonadectomy on intracellular Ca²⁺ mobilization in rat platelets

		Ca mobilization (nmol/10 ⁸ platelets)		
		Control	Aspirin	
			10 mg/kg	20 mg/kg
Male	Intact	482±26 (100%)	297±37* ¹ (62%)	240±32* ¹ (49%)
	Castration	327±27* ² (100%)	229±22 (70%)	172±11* ¹ (53%)
Female	Intact	300±34* ² (100%)	227±16 (76%)	209±14* ¹ (70%)
	Ovariectomy	291±28* ² (100%)	178±11* ¹ (61%)	170±5* ¹ (58%)

The values are the mean±S.E. (n=5). Values in parentheses represent % of control. Platelets were stimulated by thrombin (1 U/ml) in the presence of external Ca²⁺ (1 mM). *¹: Significant difference from the control value of each sex (P<0.05). *²: Significant difference from the value of intact control male (P<0.05).

Table 2. Influence of gonadectomy on TXB₂ production in rat platelets

		TXB ₂ production (pmol/10 ⁸ platelets)		
		Control	Aspirin	
			10 mg/kg	20 mg/kg
Male	Intact	422±33 (100%)	161±34* ¹ (38%)	137±25* ¹ (33%)
	Castration	199±23* ² (100%)	124±33 (62%)	84±31* ¹ (42%)
Female	Intact	214±25* ² (100%)	130±25 (61%)	93±30* ¹ (44%)
	Ovariectomy	173±18* ² (100%)	118±37 (68%)	40±26* ¹ (23%)

The values are the mean±S.E. (n=5). Values in parentheses represent % of control. Platelets were stimulated by thrombin (1 U/ml) in the presence of external Ca²⁺ (1 mM). *¹: Significant difference from the control value of each sex (P<0.05). *²: Significant difference from the value of intact control male (P<0.05).

stimulated platelets. Castration decreased TXB₂ production in male platelets to the same level as intact female platelets, and the inhibitory effect of aspirin on TXB₂ production was weakened by castration. Ovariectomy did not influence TXB₂ production, but enhanced the inhibitory effect of aspirin.

Discussion

It has been demonstrated that men and male animals suffer from thrombosis more frequently than women and females (1, 12). Moreover, in humans and experimental animals, male platelets were aggregated more strongly by several stimulators than female platelets (13–15). The sex differences in platelet functions are concerned with the sex differences in the risk of thrombus formation and in platelet aggregation. In randomized clinical trials (3), it was observed that aspirin was efficacious for men with threatened stroke. Ca²⁺ has an important role in platelet functions, and it was suggested that aspirin modulated TXA₂ production in platelets (16). TXA₂ is a potent platelet aggregator, and it was reported that TXA₂ induced Ca²⁺ mobilization in human platelets (17). We thought that it was worthwhile to investigate the relationship between Ca²⁺ mobilization and the sex difference in the antiplatelet effect of aspirin.

In our previous work (8), aspirin at a dose of 10 and 20 mg/kg inhibited rat platelet aggregation, and aspirin at a dose of 5 mg/kg inhibited malondialdehyde production, as an index of TXA₂ production. So, we used the doses of 5, 10 and 20 mg/kg in this work, and studied the inhibitory effect of aspirin on Ca²⁺ mobilization and TXA₂ production in rat platelets of both sexes.

Ca²⁺ mobilization in thrombin-stimulated platelets was greater in male rats than in females. TXA₂ production in male platelets was greater than that in female platelets, and it agreed with the result of Kelton et al. (18). Aspirin decreased Ca²⁺ mobilization in platelets, and this inhibitory effect was stronger in males than in females. Moreover, TXA₂ production in male platelets was greater than that in female platelets. It was reported that TXA₂ acted as a second messenger for Ca²⁺ mobilization in human platelets (19–

21), and aspirin and indomethacin decreased the rise in Ca²⁺ mobilization (20, 21). Rink et al. (7, 11, 17) suggested that in human platelets stimulated with thrombin and arachidonic acid, TXA₂ increased the intracellular Ca²⁺ mobilization mainly by the Ca²⁺ influx and partly by the internal Ca²⁺ discharge and that aspirin modulated Ca²⁺ mobilization. In this study, aspirin inhibited Ca²⁺ mobilization more strongly in the presence of external Ca²⁺ than in its absence. Though there were some differences in experimental conditions, our results supported Rink's finding that aspirin inhibited Ca²⁺ mobilization at the site of Ca²⁺ influx. The sex difference in the effect of aspirin on intracellular Ca²⁺ mobilization seems to be correlated with the sex difference in the effect of aspirin on TXA₂ production.

The intracellular Ca²⁺ concentration in aspirin-treated platelets was of the same level as that in the control rat platelets, and there was no sex difference in the intracellular Ca²⁺ concentration of rat platelets in the resting state (before stimulation by thrombin). Aspirin did not decrease the intracellular Ca²⁺ concentration in rat platelets in the resting state.

There was a difference between the inhibitory effect of aspirin on Ca²⁺ mobilization and that on TXA₂ production in this study. Many workers have been proposed mechanisms for Ca²⁺ mobilization in thrombin-stimulated platelets: for example, TXA₂ acts like a Ca²⁺ ionophore (19), the cleavers of inositol-phosphatides acts as second messengers for Ca²⁺ mobilization (22), and Ca²⁺ mobilization was independent of TXA₂ production (23). In this study, aspirin partly inhibited Ca²⁺ mobilization in thrombin-stimulated platelets, but completely abolished TXA₂ production. Therefore, it is suggested that TXA₂ is only one of many factors of Ca²⁺ mobilization induced by thrombin.

It was reported that testosterone enhanced rat platelet aggregation *in vitro* (14, 15). Our previous report (8) showed that castration decreased malondialdehyde production in rat platelets. Pilo et al. (15) suggested that the potentiating effect of testosterone was abolished by indomethacin.

However, Chang et al. (24, 25) suggested that sex hormones did not stimulate platelet cyclooxygenase in male rats. In this study, castration decreased Ca^{2+} mobilization and TXA_2 production in rat platelets, but ovariectomy did not influence them. Previously, we suggested that platelet cyclooxygenase activity was more strongly influenced by castration than by ovariectomy (8). Aspirin efficiently suppressed Ca^{2+} mobilization and TXA_2 production in male rat platelets. The inhibitory effect of aspirin on Ca^{2+} mobilization was consistent with its effects on platelet aggregation and platelet TXA_2 production. It is supposed that testosterone modulates the platelet cyclooxygenase activity in platelets by some unknown mechanism and influences Ca^{2+} mobilization.

In conclusion, it is suggested that the sex difference in TXA_2 production causes the sex difference in the antiplatelet effect of aspirin and participates in the sex difference in Ca^{2+} mobilization in rat platelets.

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