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Effects of magnesium lithospermate B on aggregation and 5-HT release in rabbit washed platelets[1]

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KEY WORDS magnesium lithospermate B; platelet aggregation; serotonin; thrombin; arachidonic acids; thromboxane B_2; calcium

ABSTRACT

AIM: To study the effects and mechanism of magnesium lithospermate B(MLB) on rabbit platelet aggregation and 5-HT release. **METHODS**: The platelet aggregation was determined by Born's method. Release of serotonin (5-HT) and formation of thromboxane A_2 (TXA_2) were measured by fluorophotometry and radioimmunoassay (RIA) respectively. Cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in platelets was measured by Fura 2-AM fluorescence technique. **RESULTS**: In washed platelets, thrombin (200 U/L) or arachidonic acid (AA) (30 μmol/L)-induced aggregation was inhibited by MLB 50-800 mg/L in a concentration-dependent manner. In addition, MLB had more inhibitory effects on platelet aggregation in the absence of extracellular calcium with IC_{50} of 102 mg/L than in the presence of $CaCl_2$ 1 mmol/L with IC_{50} of 194 mg/L. MLB concentration-dependently decreased the thrombin-activated release of 5-HT, whereas it did not affect the formation of TXA_2 in platelets. Furthermore, MLB not only inhibited the rise of $[Ca^{2+}]_i$ in thrombin stimulated platelets, but decreased the $[Ca^{2+}]_i$ in resting platelets. **CONCLUSION**: MLB inhibited the aggregation and 5-HT release in rabbit platelets and it is probably by attenuating intracellular calcium concentration.

INTRODUCTION

Radix Salviae Mitiorrhizae has been reported to show vasodilatory, hypotensive, and anticogulant activities, and to have a beneficial effect in patients with ischemia, and stasis diseases[1]. Magnesium lithospermate B (MLB) is a biologically active component isolated from Radix Salviae Mitiorrhizae aqueous extract[2,3]. It has been shown that MLB has the activities of anti-oxidation[3,4], anti-inflammation, protection of CCl_4-injured hepatocytes[4], and improvement of renal failure[5]. However, as yet there have been few published reports correlating the anti-aggregatory effects of MLB. Therefore, this experiment was undertaken to study the effects of MLB on aggregation and 5-HT release in rabbit platelet.

MATERIALS AND METHODS

Animals Rabbits, weighing 2.0-3.0 kg, ♂, were supplied by the Department of Laboratory Animals, Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Drugs and reagents MLB was supplied by Department of Biotechnology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Thrombin was supplied by Shanghai Hua-Shan Hospital. All of the agents and aspirin (Asp) were dissolved in distilled water and freshly prepared. Arachidonic acid (AA, Fluka, Buchs, Switzerland) dissolved in ethanol, was diluted to 0.5 % solution with 1 % Na_2CO_3. TXB_2 RIA kit was purchased from Institute of Dong-Ya Immunotechnology, Beijing, China. Fura 2-AM was the product of Sigma Chemical Co. Egtazic acid was from TCI Co, Tokyo, Japan. Other reagents were AR.

Preparation of the rabbit washed platelets[6] Rabbit blood from inferior venacava anticoagulated with 1/6 (v/v) acid citrate-dextrose solution (ACD, sodium citrate 85, citrate acid 71, glucose 110 mmol/L), was centrifuged at 200 × *g* for 15 min to get platelet-rich plasma. The latter was spun again (800 × *g*, 20 min) and suspended in calcium-free HEPES buffer (NaCl 145, KCl

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5, $MgSO_4$ 1, glucose 5, and HEPES 10 mmol/L, pH 7.4). The washed platelet counts were adjusted to 4×10^8/L.

Measurement of platelet aggregation The aggregation of the washed platelets induced by thrombin 200 U/L or AA 30 μmol/L, was measured by Born's method[7] with MPG-3D model aggregometer (Shanghai Institute of Gao-Ji Applied Technology) with or without $CaCl_2$ 1 mmol/L. MLB or Asp was pre-incubated in the washed platelet at 37 ℃ for 5 min before determination. Results were expressed as maximal-aggregation and percentage of inhibition *vs* control values.

Assay for 5-HT released in the washed platelet[8] After being aggregated by thrombin 200 U/L with $CaCl_2$ 1 mmol/L, the platelets were centrifuged at 3500 × *g* for 15 min. The content of 5-HT in the supernate 0.4 mL was determined with fluorophotometry (Model 651-10s, Hitachi, Japan).

RIA for TXB_2 formation in the washed platelet[9] The washed platelet 1 mL containing MLB or Asp was incubated with $CaCl_2$ 1 mmol/L at 37 ℃ for 20 min. Then AA 30 mmol/L was added and the mixture was kept in the warm-bath for 5 min. The reaction was terminated by 10 % methane acid 120 μL, and the solution was extracted by ethyl acetate 2.5 mL twice. The content of TXB_2 in the merged organic phase was assayed using RIA kit.

Measurement of cytosolic Ca^{2+} Intracellular Ca^{2+} concentration of platelets was measured using Fura 2-AM (excitation 340 nm and 380 nm, emission 490 nm) with a fluorophotometry (Model 651-10s, Hitachi, Japan) at room temperature. The ratio of the measured fluoresence values at 340 and 380 nm excitation ($ratio_{F340/F380}$) was used to express the cytosolic calcium concentration[10]. In the presence of egtazic acid 3 mmol/L, the $[Ca^{2+}]_i$ in platelets exposed to MLB for 2 min was measured with or without thrombin 200 U/L.

Statistical analysis All data were expressed as $\bar{x} \pm s$, *P* value was evaluated by *t* test. Values of IC_{50} were calculated and the difference between them was tested by Logit method.

RESULTS

MLB inhibited thrombin-induced aggregation MLB (100, 200, 300, 400, 800 mg/L) inhibited rabbit washed platelet aggregation induced by thrombin 200 U/L with $CaCl_2$ 1 mmol/L in a concentration-dependent manner. Similar inhibitory effects were obtained in platelets in the absence of $CaCl_2$. The inhibitory rates were (23 ± 7) %, (41 ± 14) %, (82 ± 13) %, (89 ± 4) %, and (92 ± 6) % when MLB 50, 100, 200, 300, and 400 mg/L were used respectively (Tab 1).

Tab 1. Effects of magnesium lithospermate B (MLB) on washed platelet aggregation induced by thrombin 200 U/L with or without $CaCl_2$ 1 mmol/L. $n = 6$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control.

Drug mg/L	With Ca^{2+} Aggregation /%	With Ca^{2+} Inhibition /%	Without Ca^{2+} Aggregation /%	Without Ca^{2+} Inhibition /%
Control	84 ± 4	– –	38 ± 7	– –
Asp 20	52 ± 6^c	39 ± 7	10 ± 5^c	72 ± 12
MLB				
50	– –	– –	32 ± 8^a	23 ± 7
100	76 ± 12^a	10 ± 11	23 ± 6^c	41 ± 14
200	44 ± 13^c	48 ± 16	7 ± 6^c	82 ± 13
300	9 ± 6^c	89 ± 8	4 ± 2^c	89 ± 4
400	8 ± 6^c	90 ± 8	3 ± 2^c	92 ± 6
800	4 ± 1^c	96 ± 2	– –	– –

Effects of MLB on AA-activated aggregation After a 5-min exposure to MLB 50, 100, 200, 400, 600, 800 mg/L with $CaCl_2$ 1 mmol/L, the platelet aggregation caused by AA 30 μmol/L was (53 ± 2) %, (48 ± 2) %, (42 ± 1) %, (40 ± 2) %, (36 ± 2) %, and (29 ± 3), % respectively, while the control group was (59 ± 3) % (Tab 2).

Tab 2. Effects of MLB on washed platelet aggregation induced by AA 30 μmol/L with $CaCl_2$ 1 mmol/L. $n = 5$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Drug/mg·L^{-1}	Aggregation/%	Inhibition/%
Control	59 ± 3	– –
Asp 20	31 ± 5^c	47 ± 8
MLB		
50	53 ± 2^b	10 ± 4
100	48 ± 2^c	19 ± 5
200	42 ± 1^c	29 ± 3
400	40 ± 2^c	31 ± 5
600	36 ± 2^c	40 ± 4
800	29 ± 3^c	51 ± 5

Comparison of effects of extracellular calcium on aggregation using IC_{50} In the presence of various concentrations of MLB, thrombin-induced platelet

aggregation was inhibited with or without extracellular calcium. The IC_{50} were 102 mg/L (95 % confidence was from 80 to 129 mg/L) and 194 mg/L (95 % confidence was from 126 to 299 mg/L) in the absence or presence of $CaCl_2$ 1 mmol/L, respectively. The difference between two "IC_{50}" was very significant ($P < 0.01$), however no difference was found between the slopes. On the other hand, the effect of MLB was much more potent on platelet aggregation induced by thrombin than that activated by AA. The maximal inhibitory effect of MLB on thrombin-induced aggregation was (96 ± 2) %, while it was (51 ± 5) % on AA-activated aggregation with MLB 800 mg/L (Fig 1).

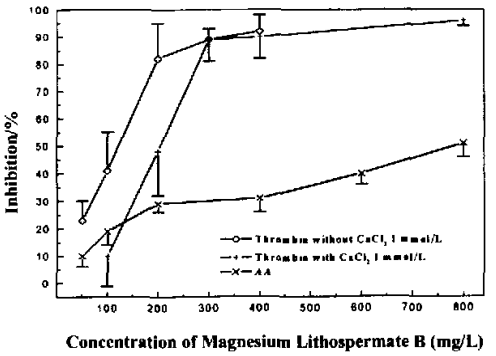

Fig 1. Concentration-inhibition curve of MLB on thrombin (200 U/L)-or AA-30 μmol/L activated aggregation in washed platelets with or without $CaCl_2$.

Effects of MLB on TXB_2 formation in washed platelets MLB 200 and 800 mg/L did not affect the TXB_2 formation stimulated by thrombin ($P > 0.05$ compared with control group). In contrast, Asp 20 mg/L lowered the level of TXB_2 markedly, the value of TXB_2 was (30 ± 4) mg/L ($P < 0.01$ *vs* control) (Tab 3).

Tab 3. Effects of MLB and Asp on TXB_2 production induced by AA 30 μmol/L in the rabbit washed platelets. $n = 5$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control.

Group	TXB_2 (μg/L)	Inhibition (%)
Control	37.9 ± 1.2	– –
MLB 200 mg/L	37.8 ± 0.8[a]	0
MLB 800 mg/L	36.6 ± 1.1[a]	3
Asp 20 mg/L	30 ± 4[c]	20

Effects of MLB on 5-HT release from the washed platelets Thrombin 200 U/L evoked, release rate of platelets in control (normal saline) was 49 %. In the presence of MLB 100, 200, 800 mg/L, the platelet 5-HT release was decreased, the inhibitory rates were 5 % ($P > 0.05$), 29 % ($P < 0.01$), and 50 % ($P < 0.01$), respectively (Tab 4).

Tab 4. Effects of MLB on 5-HT released from the rabbit washed platelets induced by thrombin 200 U/L. $n = 7$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control.

Group	5-HT (mg/L)	Release Rate (%)	Inhibition (%)
Control	7.8 ± 0.99	– –	– –
Thrombin + NS	3.8 ± 0.49	49	– –
Thrombin + MLB 100 mg/L	3.6 ± 0.52[a]	46	5
Thrombin + MLB 200 mg/L	2.7 ± 0.53[c]	35	29
Thrombin + MLB 800 mg/L	1.9 ± 0.37[c]	24	50

Effects on cytosolic Ca^{2+} in washed platelets In the presence of egtazic acid 3 mmol/L , the ratio$_{F340/F380}$ of resting level of $[Ca^{2+}]_i$ in platelets was 0.99 ± 0.14. After being exposed to MLB 8, 24, and 80 mg/L, the ratio$_{F340/F380}$ of $[Ca^{2+}]_i$ was 0.67 ± 0.06, 0.52 ± 0.05, and 0.34 ± 0.04, respectively .

After being stimulated by thrombin 200 U/L, the rise in $[Ca^{2+}]_i$ of platelets was enhanced from the resting level to 1.85 ± 0.22. MLB 8, 24, and 80 mg/L decreased the ratio$_{F340/F380}$ of $[Ca^{2+}]_i$ elevation to 0.95 ± 0.11, 0.66 ± 0.09, and 0.41 ± 0.04, respectively (Fig 2).

DISCUSSION

In this paper the *in vitro* experiments showed that MLB inhibited the rabbit washed platelet aggregation induced by thrombin or AA, the results of which are similar to the results of *in vivo* study (data not published). It is known that thrombin-induced rise in $[Ca^{2+}]_i$ in platelets is a combination of Ca^{2+} release from $[Ca^{2+}]_i$ store and influx of extracellular calcium[11]. In the absence of extracellular calcium, MLB not only inhibited platelets aggregation and the rise of $[Ca^{2+}]_i$ induced by thrombin in a concentration-dependent manner, but decreased the

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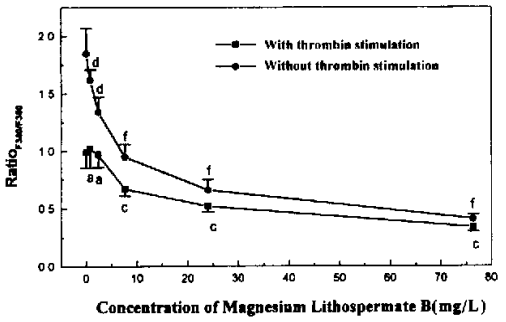

Fig 2. Effects of MLB 0.8, 2.4, 8, 24, 76 mg/L on ratio$_{F340/F380}$ of $[Ca^{2+}]_i$ of platelets in the presence of egtazic acid 3 mmol/L. $n = 4$. $^aP > 0.05$, $^cP < 0.01$ *vs* the group without thrombin; $^dP > 0.05$, $^fP < 0.01$ *vs* the group with thrombin 200 U/L.

level of calcium in resting platelets. These results suggested that the antiplatelet effect of MLB was closely related to intracellular calcium of platelets.

TXA_2, as a strong aggregatory agent, causes mobilization of $[Ca^{2+}]_i$ from storage sites in platelets[12]. In the present study, it was indicated that MLB had more selective inhibition on thrombin activation than the AA-evoked platelet aggregation when comparing the inhibitory curves by AA and by thrombin. In addition, the RIA experiments showed that MLB had no effect on TXA_2 formation. This indicated that MLB had fewer effects on the AA metabolism in platelets.

According to the current hypothesis, the release of 5-HT is closely associated with the $[Ca^{2+}]_i$ level in platelets and platelet aggregation function[13], but not to an agonist of rabbit platelet aggregation[14]. The inhibition on 5-HT release suggested that MLB might affect the process of platelet release and be related to cytosolic calcium level.

In summary, MLB strongly inhibited the platelet aggregation in the absence of extracellular calcium, which suggested that MLB probably attenuated the intracellular calcium. More evidence is needed to evaluate the mechanism.

REFERENCES

1 Yue RS. The new development of research on the pharmacological activities of *Salviae mitiorrhizae* Bga. New Chin Med 1991; 11: 54-6.
2 Tanaka T, Morimoto S, Nonaka G, Nishioka I, Yokozawa T, Hae YC, *et al*. Magnesium and Ammonium-Potassium Lithospermates B, the active principles having an uremia-preventive effect from *Salvia miltiorrhiza*. Chem Pharm Bull 1989; 37: 340-4.
3 Xu YM, Xuan LJ. The polyphenoles of *Salvia miltiorrhiza* and their pharmacological activities. Ye Y, editor. Progress in drug development from medicinal plants: 169-72. Proceeding of UNESCO Regional Symposium on Drug Development form Medicinal Plants; 1996 Oct 25-27; Hangzhou, China.
4 Liu P. Effects of MLB on cyclooxygenase activity on rat liver, adherent cells and enzyme's product. China J Chin Mater Med 1994; 19: 110-3.
5 Yokozawa T, Hae YC, Tae WL, Oura H, Tanaka T, Nonaka G, *et al*. Contribution of prostaglandins to the renal responses to magnesium lithospermate B isolated from *Salviae miltiorrhizae* Radix. Chem Pharm Bull 1989; 37: 1568-71.
6 Li BY, Bai Y, Li GZ, Li WH, Makoto K. Effects of MK-447 on thrombin-induced aggregation, secretion of ATP and $[Ca^{2+}]_i$ mobilization in rabbit platelet. Acta Pharmacol Sin 1995; 16: 108-13.
7 Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962; 194: 927-9.
8 Wan HY, Chen H, Xi XD, Ruan CG. Fluorophoto for platelet 5-HT. Jiangsu Med J 1985; 12: 24-5.
9 Zhang B, Long K, Jiang YY. Effects of glaucocalyxin A on PAF biosynthesis and arachidonic acid metabolism in washed rabbit platelets. Chin J Pharmacol Toxicol 1992; 6: 52-5.
10 Wang YP, Oike M, Ito Y. Effects of superoxide anion on intracellular Ca^{2+} concentration in rabbit pulmonary arterial smooth muscle cells. Acta Pharmacol Sin 1999; 20: 10-4.
11 Ware JA, Johnson PC, Smith M, Saizman EW. Effects of common agonist on cytoplasmic ionized calcium concentration in platelets. J Clin Invest 1986; 77: 878-86.
12 Gerrard JM. The possible role of biological calcium ionophores in platelet activation. Thromb Haemost 1983; 50: 92.
13 Li BY, Li WH. Effects of 5-HT released from platelets on thrombin-induced aggregation and ATP release in rabbit platelets *in vitro*. Acta Pharmacol Sin 1998; 19: 383-6.
14 Li BY, Bai Y, Li GZ. Enhancement of ADP-induced aggregation by 5-HT in rabbit platelet. Acta Pharmacol Sin 1998; 19: 58-62.

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丹酚酸 B 镁盐对兔洗涤血小板聚集和 5-HT 释放反应的影响[1]

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关键词 丹酚酸 B 镁盐；血小板聚集；血清素；凝血酶；花生四烯酸类；血栓素 B_2；钙

目的：研究丹酚酸 B 镁盐（magnesium lithospermate B）对血小板聚集和 5-HT 释放功能的影响及其作用机制．**方法**：比浊法评价兔洗涤血小板的聚集反应；荧光分光光度法和放射免疫法观察血小板的 5-HT 与 TXA_2 的释放；用 Fura 2 荧光探针测定血小板胞内钙浓度变化．**结果**：丹酚酸 B 镁盐 50 – 800 mg/L 对凝血酶或花生四烯酸诱导的血小板聚集反应有剂量依赖性的抑制作用，当体系无外 Ca^{2+} 存在，丹酚酸 B 镁盐的抑制作用增强，IC_{50} 为 102 mg/L．药物对血小板的 5-HT 释放也有剂量依赖性的抑制作用，但对 AA 激发的 TXA_2 释放无影响．同时，丹酚酸 B 镁盐对凝血酶激发的胞内钙升高有明显的抑制作用，并降低静息血小板 $[Ca^{2+}]_i$．**结论**：丹酚酸 B 镁盐对体外洗涤血小板的聚集和 5-HT 释放反应有明确的抑制作用，可能与影响血小板胞内 Ca^{2+} 浓度的机制有关．

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