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Inhibitory effects of sodium quercetin monosulfate on pig platelet aggregation induced by thrombin[1]

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KEY WORDS blood platelets; quercetin; platelet aggregation; calcium; protein kinase C; actins; thrombin

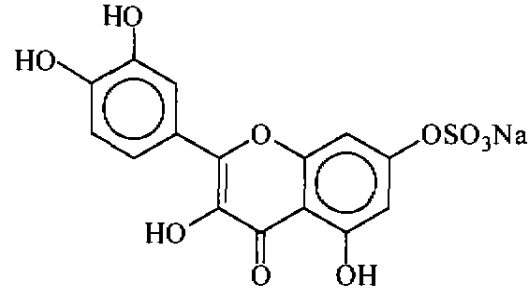
ABSTRACT

AIM: To study the inhibitory effects of sodium quercetin monosulfate (SQMS) on pig platelet aggregation induced by thrombin. **METHODS:** Platelet aggregation was analyzed by turbidimetry. Cytosolic free calcium concentration ($[Ca^{2+}]_i$) was determined by Fura-2 fluorescence. Activity of protein kinase C (PKC) was assayed by incubating PKC with histone III S and [γ-^{32}P] ATP. The cytoskeletal proteins were precipitated by Triton X-100 and separated by SDS-PAGE. **RESULTS:** SQMS inhibited the platelet aggregation induced by thrombin 500 U·L^{-1} with IC_{50} 132 (50 - 347) μmol·L^{-1}. SQMS inhibited Ca^{2+} influx in blood platelets induced by thrombin 500 U·L^{-1} in the presence of extracellular Ca^{2+} 1 mmol·L^{-1} with IC_{50} 20 (9 - 46) μmol·L^{-1}; SQMS inhibited the internal Ca^{2+} release in the absence of extracellular Ca^{2+}. SQMS also decreased $[Ca^{2+}]_i$ level in quiescent blood platelets. SQMS (10 - 160 μmol·L^{-1}) inhibited the activity of cytosolic PKC from blood platelets in a concentration-dependent manner, but had no effect on membrane PKC. SQMS (20 - 80 μmol·L^{-1}) inhibited the actin polymerization induced by thrombin 500 U·L^{-1} in blood platelets in a concentration-dependent manner. **CONCLUSION:** SQMS inhibited pig platelet aggregation induced by thrombin and its mechanism might be due to its inhibitions of Ca^{2+} influx, internal Ca^{2+} release, PKC activity, and actin polymerization.

INTRODUCTION

Flavonoids are found in many plants. Various pharmacological activities of flavonoids have been studied extensively on blood platelets[1-4], but the mechanism of flavonoids on blood platelet remains unclear.

Platelet aggregation induced by thrombin is considered to be a very complicated procedure. The cytosolic free calcium concentration ($[Ca^{2+}]_i$), protein kinase C (PKC), and redistribution of filamentous actin (F-actin) are thought to play important roles in this activated procedure[5-9]. In the present report, we described the effects of sodium quercetin monosulfate (SQMS) on thrombin-induced platelet aggregation, $[Ca^{2+}]_i$, on the activities of cytosolic and membrane PKC, and on actin polymerization in pig platelets.



Sodium quercetin monosulfate (SQMS)

MATERIALS AND METHODS

Bovine thrombin, Tris, HEPES, RPMI-1640, egtazic acid (EGTA), Fura-2-acetoxy methylester

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(Fura 2-AM), quercetin, phosphatidylserine (PS), diolein, histone Ⅲ S, bovine serum albumin (BSA) (Sigma); Triton X-100 (Merck), [γ-^{32}P]ATP (Yahui Biomedical Technology Co Ltd, Beijing). SQMS was synthesized with quercetin and concentrated H_2SO_4 by Prof MO Li-Er (the Department of Chemistry). All other chemicals were of AR.

Pig blood was collected in plastic tubes and anticoagulated with 0.15 volume of ACD (trisodium citrate 86, glucose 111, citric acid 53 mmol·L^{-1}) or 0.1 volume of EDTA buffer (NaCl 120, Tris 50, edetic acid 50 mmol·L^{-1}, pH 7.4).

Platelet aggregation Platelet aggregation assay was performed as Huang C, *et al* reported[10].

Cytosolic free calcium Cytosolic free calcium assay was performed as Huang C, *et al* reported[10].

Cytoskeletal proteins Cytoskeletal proteins assay was performed as Huang C, *et al* reported[10].

Partial purification and assay of PKC Partial purification and assay of PKC were performed as Kang TB, *et al* reported[11].

Data were expressed as $\bar{x} \pm s$ and analyzed by t test.

RESULTS

Effects of SQMS on thrombin-induced platelet aggregation Stimulation of platelets with thrombin (500 U·L^{-1}) resulted in 82 % ± 12 % ($n = 4$) of platelets aggregation. SQMS inhibited the platelet aggregation induced by thrombin with IC_{50} 132 (50 – 347) μmol·L^{-1}.

Effects of SQMS on thrombin-induced $[Ca^{2+}]_i$ increase The fluorescence of platelets was relatively stable in 1 h with no drug in the presence of extracellular Ca^{2+} 1 mmol·L^{-1}. Thrombin 500 U·L^{-1} stimulated the increase of $[Ca^{2+}]_i$ in the absence or presence of extracellular Ca^{2+} 1 mmol·L^{-1}. The action of thrombin in the presence of extracellular Ca^{2+} 1 mmol·L^{-1} was inhibited by SQMS with IC_{50} 20 (9 – 46) μmol·L^{-1}; SQMS also had inhibitory effects on internal Ca^{2+} release in the absence of extracellular Ca^{2+} (Tab 1).

SQMS also decreased $[Ca^{2+}]_i$ level in quiescent blood platelets in the absence or presence of extracellular Ca^{2+} 1 mmol·L^{-1} (Tab 2).

Tab 1. Effect of SQMS on $[Ca^{2+}]_i$ in pig platelets stimulated by thrombin 500 U·L^{-1} in the presence or absence of extracellular calcium 1 mmol·L^{-1}. $n = 4$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* thrombin alone.

SQMS/ μmol·L^{-1}	Thrombin/ U·L^{-1}	$[Ca^{2+}]_i$/nmol·L^{-1} Ca^{2+} 1 mmol·L^{-1}	EGTA 1 mmol·L^{-1}
0	0	247 ± 7	43.3 ± 2.4
0	500	651 ± 26	83 ± 3
10	500	476 ± 24^b	
20	500	357 ± 23^b	48 ± 5^b
40	500	166 ± 20^c	31 ± 4^c

Tab 2. Effect of SQMS on $[Ca^{2+}]_i$ level in quiescent pig platelets in the presence or absence of extracellular calcium 1 mmol·L^{-1}. $n = 4$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

SQMS/ μmol·L^{-1}	$[Ca^{2+}]_i$/ nmol·L^{-1} Ca^{2+} 1 mmol·L^{-1}	EGTA 1 mmol·L^{-1}
0	328 ± 7	35.0 ± 1.2
20	240 ± 25^c	23.7 ± 0.8^b
40	167 ± 6^c	17.6 ± 1.2^c
80	126.7 ± 2.8^c	15.4 ± 1.6^c
160	109.2 ± 2.3^c	

Effects of SQMS on cytosolic PKC and membrane PKC SQMS (10 – 160 μmol·L^{-1}) inhibited the activity of cytosolic PKC from blood platelets in a concentration-dependent manner, but had no effect on membrane PKC (Tab 3).

Tab 3. Effects of SQMS on the activity of cytosolic PKC and membrane PKC from pig platelets. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Treatment SQMS μmol·L^{-1}	Protein kinase C/Bq Cytosol	Membrane
0	68 ± 17	21.5 ± 0.8
10	29 ± 4^b	22 ± 17^a
20	33 ± 5^b	17.2 ± 1.0^a
40	13 ± 3^c	25 ± 3^a
80	12 ± 4^c	24.2 ± 1.7^a
160	3.6 ± 1.2^c	20.4 ± 2.1^a

Label: [γ-^{32}P]ATP

Effect of SQMS on the actin polymerization induced by thrombin in blood platelet

Thrombin 500 U · L^{-1} stimulated the increase of F-actin. SQMS (20 – 80 μmol·L^{-1}) inhibited the actin polymerization induced by thrombin in blood platelets in a concentration-dependent manner (Fig 1, Tab 4).

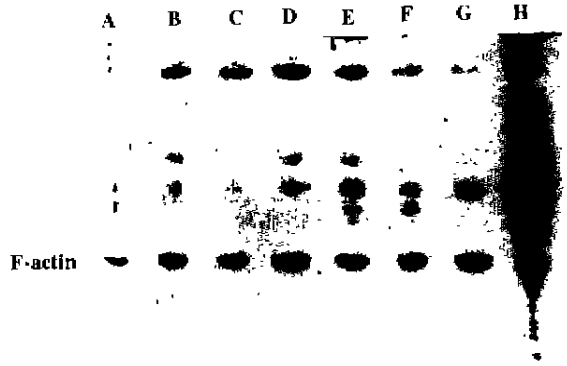

Fig 1. Effect of SQMS on actin polymerization induced by thrombin in pig platelets (SDS-PAGE of Triton-insoluble cytoskeletons). A) SQMS 320 μmol·L^{-1} + thrombin; B) SQMS 160 μmol·L^{-1} + thrombin; C) SQMS 80 μmol·L^{-1} + thrombin; D) SQMS 40 μmol·L^{-1} + thrombin; E) SQMS 20 μmol·L^{-1} + thrombin; F) Control; G) Thrombin; H) Whole platelets.

Tab 4. Effect of SQMS on actin polymerization induced by thrombin 500 U·L^{-1} in pig platelets. $n = 4$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* thrombin alone.

SQMS/ μmol·L^{-1}	Thrombin/ U·L^{-1}	F-actin/ % of total actin
0	0	30 ± 4
0	500	42 ± 6
20	500	35 ± 5^b
40	500	32 ± 6^b
80	500	27 ± 4^c
160	500	25 ± 5^c
320	500	25 ± 6^b

DISCUSSION

The results demonstrated that SQMS inhibited thrombin-induced platelet aggregation. These and other evidence [1-4] indicate that some flavonoids may be a type of anti-platelet agents.

Ca^{2+} is thought to play important roles in platelet aggregation, and PKC is the important regulator of platelet aggregation[5-8]. Ca^{2+} and PKC play a synergistic-action in thrombin-induced platelet aggregation[6]. SQMS inhibited Ca^{2+} influx and internal Ca^{2+} release in thrombin-induced platelets; SQMS also decreased $[Ca^{2+}]_i$ level in quiescent blood platelets. Ca^{2+} influx is a major pathway for $[Ca^{2+}]_i$ in thrombin-induced platelets or quiescent platelets. IP_3 is a second messenger for intracellular Ca^{2+} release. These results indicated that SQMS might affect IP_3 level directly. SQMS inhibited the activity of cytosolic PKC from blood platelets, but had no effect on membrane PKC. This may be due to distribution of PKC in platelets. Whether it may be related to the translocation of PKC will be further studied. The effects of SQMS on $[Ca^{2+}]_i$ and activity of cytosolic PKC could be related to its inhibition of platelet aggregation. These suggest that SQMS inhibits platelet aggregation induced by thrombin due to the inhibition of Ca^{2+} influx, internal Ca^{2+} release, and PKC activity.

Resting platelets have most of their actin (60 % – 70 %) in the unpolymerized form, G-actin. Stimulation of platelets with various agonists, such as thrombin induces a rapid increase in cytoskeletal actin[9,12]. Although the molecular mechanism of actin polymerization induced by thrombin is unclear, the redistribution of F-actin was involved in platelet aggregation. The results showed that SQMS inhibited the actin polymerization induced by thrombin in blood platelets. Chen RY, *et al* reported that PKC might play an important role in actin polymerization[7]. Our results also supported this view. The effects of SQMS on thrombin-induced actin polymerization could be related to its inhibition on platelet aggregation. The results suggested that inhibitory effect of SQMS on platelet aggregation induced by thrombin was due to its inhibition of actin polymerization.

In summary, this study demonstrated that SQMS inhibited platelet aggregation induced by thrombin and its molecular mechanism was due to its inhibition on Ca^{2+} influx, internal Ca^{2+} release, PKC activity, and actin polymerization. This work primarily provides evidence that helps to clarify the molecular mechanism of the antiplatelet actions of SQMS.

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槲皮素单硫酸酯钠盐对凝血酶诱导的猪血小板聚集的抑制作用[1]

R 973

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关键词 血小板；槲皮素；血小板聚集；钙；蛋白激酶 C；肌动蛋白类；凝血酶

目的：研究槲皮素单硫酸酯钠盐（SQMS）对凝血酶诱导的猪血小板聚集的抑制作用. **方法**：用比浊法测定血小板聚集. Fura 2-AM 荧光法检测胞浆游离钙浓度（$[Ca^{2+}]_i$）. 用组蛋白 III S，[γ-^{32}P]ATP 与蛋白激酶 C（protein kinase C，PKC）酶液一起温育的方法测定 PKC 的活性. 用 SDS-PAGE 分离骨架蛋白. **结果**：SQMS 对凝血酶诱导的血小板聚集有抑制作用. SQMS 抑制凝血酶诱导的血小板胞外钙内流和胞内钙释放；SQMS 也降低静息血小板 $[Ca^{2+}]_i$. SQMS 抑制血小板胞浆 PKC 的活性，但不影响胞膜 PKC. SQMS 对凝血酶诱导的血小板肌动蛋白聚合有强的抑制作用. **结论**：SQMS 对凝血酶诱导的猪血小板聚集有抑制作用、其分子作用机制可能与其抑制血小板外钙内流，内钙释放，PKC 的活性，和肌动蛋白聚合有关.

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