ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8): 737 - 741
http://www.chinaphar.com

Inhibitory effect of disodium quercetin-7, 4′-disulfate on aggregation of pig platelets induced by thrombin and its mechanism[1]

LIU Wen, LIANG Nian-Ci[2]
(*Institute of Biochemistry and Molecular Biology, Guangdong Medical College, Zhanjiang 524023, China*)

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

ABSTRACT

AIM: To study the inhibitory effect of semi-synthesized quercetin derivatives — disodium quercetin-7,4′-disulfate (DQD) on the platelet aggregation induced by thrombin and its mechanism. **METHODS**: Platelet aggregation was analysed by turbidimetry. Cytosolic free calcium concentration ($[Ca^{2+}]_i$) was determined by Fura-2 fluorescence technique. Activity of Ca^{2+}/PL dependent 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 and separated by SDS-PAGE. **RESULTS**: DQD inhibited the platelet aggregation induced by thrombin (500 U/L), when DQD concentrations were 100, 200, and 400 μmol/L, the inhibition rates were 77 %, 86 %, and 82 % respectively. DQD inhibited Ca^{2+} influx in platelets induced by thrombin (500 U/L) in the presence of extracellular Ca^{2+} 1 mmol/L in a concentration-dependent manner (10 - 80 μmol/L); DQD also had inhibitory effect on intracellular Ca^{2+} mobilization in the absence of extracellular Ca^{2+}. DQD (10 - 160 μmol/L) inhibited the cytosolic Ca^{2+}/PL dependent PKC from platelets in a concentration-dependent manner, but had no effect on membrane PKC. DQD (20 - 200 μmol/L) inhibited the actin polymerization induced by thrombin (500 U/L) in platelets in a concentration-dependent manner. **CONCLUSION**: DQD inhibited pig platelet aggregation induced by thrombin and its molecular mechanism was due to its inhibition of Ca^{2+} influx, intracellular Ca^{2+} mobilization, Ca^{2+}/PL dependent PKC activity, and actin polymerization.

INTRODUCTION

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a natural flavonoid. Various pharmacological activities of quercetin have been studied extensively on platelets[1-4]. But as quercetin is not water soluble, its biological utilization *in vivo* is restricted. Theoretically, quercetin can be transformed into water-soluble derivatives while maintaining its active structure 5-OH and 4′-OH[5], and the derivatives could be developed as a anti-thrombus drugs. With this in view, sodium quercetin sulfates were synthesied chemically and its anti-platelet actions were studied. Structures of quercetin derivatives were determined as sodium quercetin-7-sulfate (SQS) and disodium quercetin-7,4′-disulfate (DQD) by FAB-MS, ^{1}H-NMR, and ^{13}C-NMR[6-8]. The action of SQS on platelets has been studied[9], and in the present report, we describe the effects of DQD on thrombin-induced platelet aggregation and cytosolic free calcium concentration ($[Ca^{2+}]_i$), on the activites of cytosolic and membrane Ca^{2+}/PL dependent protein kinase C (PKC) and on actin polymerization in pig platelets.

MATERIALS AND METHODS

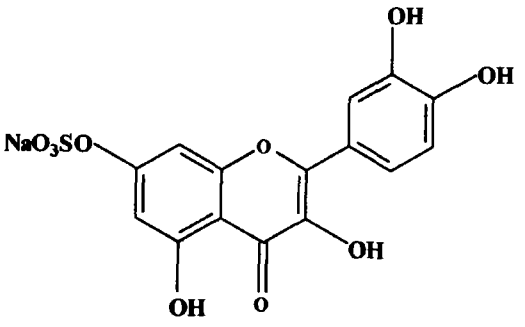
Chemicals and drugs Bovine thrombin, Tris, HEPES, RPMI 1640, egtazic acid, Fura-2-acetoxy methylester (Fura 2-AM), quercetin, phosphatidylserine (PS), diolein, histone Ⅲ S, and bovine serum albumin (BSA) (Sigma); Triton X-100 (Merck), [γ-^{32}P]ATP (Yahui Biomedical Technology Co Ltd, Beijing). All other chemicals were 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).

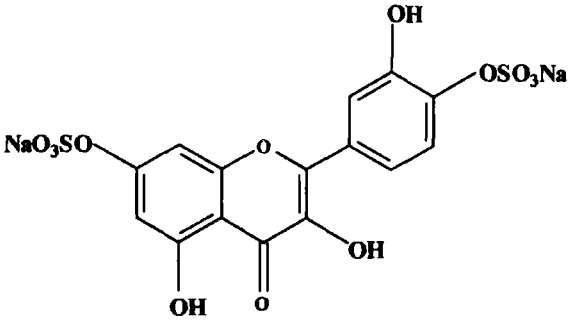
Platelet aggregation[9] Blood was centrifuged at 200 × *g* for 15 min, and the supernatant was then

[1] Project supported by Guangdong Research Foundation for Key Field, № 9306
[2] Correspondence to Prof LIANG Nian-Ci.
Phn 86-759-238-8501. Fax 86-759-228-4104.
E-mail gdmcpres@gdmc.edu.cn or wenliu@gdmc.edu.cn
Received 1999-12-06 Accepted 2000-03-03

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629



Sodium quercetin-7-sulfate (SQS)



Disodium quercetin-7,4′-disulfate (DQD)

centrifuged at 800 × *g* for 15 min. The platelets were resuspended at 2×10^{11}/L in Tyrode-HEPES buffer (NaCl 140, KCl 5, $MgSO_4$ 1, HEPES 10, glucose 10 mmol·L^{-1}, pH 7.4). Platelet aggregation was measured by a TYXN-96I (II) aggregometer (Shanghai). The platelet suspensions (0.2 mL) were incubated with DQD for 1 min, and then stimulated with thrombin 500 U/L for 5 min.

Cytosolic free calcium[9] Suspensions of platelets were incubated with Fura 2-AM. Fluorescence (λ_{ex} 340 nm; λ_{em} 500 nm) was measured at 25 ℃ using LS50B Luminescence Spectrometer (Perkin Elmer). Maximal fluorescence (F_{max}) was obtained after addition of Triton X-100 (at final concentration of 0.1 %) in the presence of $CaCl_2$ 1 mmol·L^{-1}. Minimal fluorescence (F_{min}) was obtained after addition of EGTA (at final concentration of 10 mmol·L^{-1}). Measurement of fluorescence was completed within 1 h. $[Ca^{2+}]_i$ was calculated as: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, K_d = 224 nmol·L^{-1}.

Cytoskeletal proteins[9] Platelet-rich plasma was treated with DQD for 15 min, and then stimulated with thrombin (500 U·L^{-1}) for 1 min, and lysed immediately by addition of an equal volume of a lysis buffer containing 2 % Triton X-100, EGTA 10, $MgCl_2$ 2, Tris 100, PMSF 0.2, DTT 0.2 mmol·L^{-1}, pH 7.4. The lysate was kept on ice for 2 min, and then centrifuged at 9000 *g* at 4 ℃ for 5 min to sediment what was operationally designated the Triton-insoluble cytoskeleton. The cytoskeletons were solubilized and applied to SDS-PAGE.

Partial Purification and Assay of PKC[9] Platelets were disrupted by sonication with buffer A (Tris-HCl 20, sucrose 250, EGTA 10, PMSF 0.5 mmol·L^{-1}, pH 7.5) in an ice bath. The lysate was centrifuged at 280 × *g* for 10 min; the supernatant was centrifuged at 100 000 × *g* for 1 h, and used for the assay of cytosolic PKC. To the particulate fraction was added buffer A containing 0.5 % Triton X-100, and then it was disrupted by sonication and extracted for 1 h. The extract was centrifuged at 100 000 × *g* for 1 h, and the supernatant was used for analysis of membrane PKC activity. The assay of Ca^{2+}/PL dependent PKC was performed as described by Liu *et al*.

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

RESULTS

Effect of DQD on thrombin-induced platelet aggregation Stimulation of platelets with thrombin (500 U·L^{-1}) resulted in (90 ± 3) % (n = 4) of platelet aggregation. DQD inhibited the platelet aggregation induced by thrombin. When DQD concentrations were 100, 200, and 400 μmol/L, the inhibition rates were 77 %, 86 %, and 82 % respectively (Tab 1).

Tab 1. Effect of DQD on pig platelet aggregation induced by thrombin (500 U·L^{-1}). n = 4 experiments. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

DQD/μmol·L^{-1}	Aggregation/%	Inhibition/%
0	90 ± 3	
100	21 ± 10^c	77
200	13 ± 3^c	86
400	16 ± 2^c	82

Effect of DQD on thrombin-induced $[Ca^{2+}]_i$ increase The platelets were stablized for 1 h with no drug in the presence or absence of extracellular Ca^{2+} 1 mmol/L. Thrombin 500 U/L stimulated the increase of $[Ca^{2+}]_i$ in the absence or presence of extracellular Ca^{2+}

1 mmol/L. The action of thrombin in the presence of extracellular Ca^{2+} 1 mmol/L was inhibited by DQD in a concentration-dependent manner (10 – 80 $\mu mol \cdot L^{-1}$); DQD also had inhibitory effect on intracellular Ca^{2+} mobilization in the absence of extracellular Ca^{2+} (Tab 2).

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

DQD/ $\mu mol \cdot L^{-1}$	Thrombin/ $U \cdot L^{-1}$	$[Ca^{2+}]_i$/$nmol \cdot L^{-1}$	
		Ca^{2+}/1 $mmol \cdot L^{-1}$	EGTA/1 $mmol \cdot L^{-1}$
0	0	247 ± 7	95 ± 6
0	500	651 ± 26	145 ± 11
5	500		88 ± 6^b
10	500	441 ± 26^b	76 ± 4^c
15	500		11.1 ± 0.8^c
20	500	311 ± 31^c	
40	500	264 ± 52^c	
80	500	56 ± 8^c	

Effects of DQD on the activity of cytosolic and membrane Ca^{2+}/PL dependent PKC DQD (10 – 160 $\mu mol \cdot L^{-1}$) inhibited the cytosolic Ca^{2+}/PL dependent PKC from platelets in a concentration-dependent manner, but had no effect on membrane PKC (Tab 3).

Tab 3. Effects of DQD on the activity of cytosolic and membrane Ca^{2+}/PL dependent PKC from pig platelets. Label: [γ-^{32}P] ATP. $n = 3$ experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Treatment DQD/ $\mu mol \cdot L^{-1}$	Protein kinase C/Bq	
	Cytosol	Membrane
0	68 ± 17	21.5 ± 0.8
10	53 ± 15^b	17 ± 4^a
20	41 ± 6^b	26.8 ± 1.5^a
40	29 ± 4^c	18 ± 8^a
80	25 ± 5^c	22 ± 9^a
160	17.1 ± 2.2^c	18 ± 26^a

Effect of DQD on the actin polymerization induced by thrombin in platelets Thrombin 500 U/L stimulated the increase of F-actin. DQD (20 – 200 $\mu mol \cdot L^{-1}$) inhibited the actin polymerization induced by thrombin in platelets in a concentration-dependent manner (Fig 1, Tab 4).

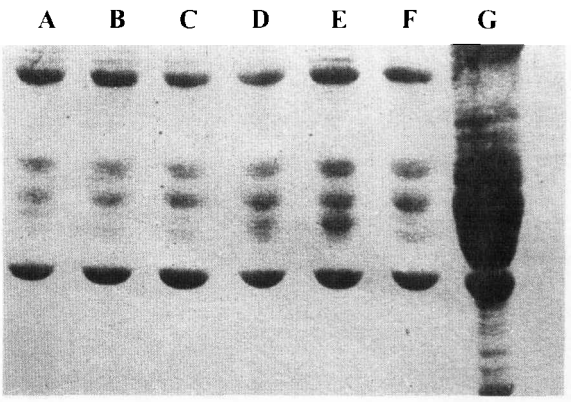


Fig 1. Effect of DQD on actin polymerization induced by thrombin in pig platelets (SDS-PAGE of triton-insoluble cytoskeletons). A) DQD 200 $\mu mol \cdot L^{-1}$ + Thrombin. B) DQD 100 $\mu mol \cdot L^{-1}$ + Thrombin. C) DQD 50 $\mu mol \cdot L^{-1}$ + Thrombin. D) DQD 20 $\mu mol \cdot L^{-1}$ + Thrombin. E) Control. F) Thrombin. G) Whole platelets.

Tab 4. Effect of DQD on actin polymerization induced by thrombin 500 $U \cdot L^{-1}$ in pig platelets. $n = 3$ experiments. $\bar{x} \pm s$. $^bP < 0.05$ *vs* thrombin alone.

DQD/ $\mu mol \cdot L^{-1}$	Thrombin/ $U \cdot L^{-1}$	F-actin/ % of total actin
0	0	24 ± 12
0	500	62 ± 5
20	500	32 ± 3^b
50	500	31 ± 8^b
100	500	28 ± 4^b
200	500	25 ± 9^b

DISCUSSION

The results demonstrated that DQD inhibited thrombin-induced platelet aggregation. These and other evidence[9] indicate that water-soluble quercetin derivatives may be a type of anti-platelet agents. In the structure of sodium quercetin sulfates, SQS has two active hydroxyl: 5-OH and 4′-OH, but DQD has only one. Theoretically, the biological activity of DQD should be weaker than SQS[9]. But the results did not indicate this characteristic which could be related to the structure and function of sodium quercetin sulfates.

Platelet aggregation induced by thrombin is considered to be a very complicated procedure. The Ca^{2+}, Ca^{2+}/PL dependent PKC, and redistribution of filamentous actin (F-actin) are thought to play important roles in

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

this activated procedure, and Ca^{2+} and Ca^{2+}/PL dependent PKC play a synergistic action in thrombin-induced platelet aggregation[10-12]. The results show that DQD inhibits Ca^{2+} influx and intracellular Ca^{2+} mobilization in thrombin-induced platelets. Ca^{2+} influx is a major pathway for $[Ca^{2+}]_i$ in thrombin-induced platelets. IP_3 is the second messenger for intracellular Ca^{2+} mobilization. These results indicate that DQD might affect IP_3 level directly. DQD inhibits the activity of cytosolic Ca^{2+}/PL dependent PKC from platelets, but has no effect on membrane PKC. Whether it may relate to the translocation of PKC, has to be further studied. Resting platelets have most of their actin (60 % - 70 %) in the unpolymerized form, G-actin. After stimulation of platelets with various agonists such as thrombin, the G-actin changes into F-actin and the actin polymerization occurs rapidly in platelets[10,13]. Although the molecular mechanism of actin polymerization induced by thrombin is unclear, Ca^{2+}, Mg^{2+}, PKC, protein-tyrosine kinase (PTK), and phosphoinositide 3-kinase (PI3-K), signal-regulated kinases (ERKs) are considered as the important regulators in this procedure, and the agents which inhibit actin polymerization can be a potential anti-platelet drugs. The results show that DQD inhibits the actin polymerization induced by thrombin in platelets. It might be related to its inhibition on Ca^{2+} influx, intracellular Ca^{2+} mobilization, and PKC. In other cells[14,15], quercetin has an inhibition on PI3-K and PTK, and the inhibition of DQD on platelet aggregation and actin polymerization could also be due to its inhibition on PI3-K and PTK.

In summary, this study demonstrated that: DQD inhibited platelet aggregation induced by thrombin and its molecular mechanism was due to its inhibition on Ca^{2+} influx, intracellular Ca^{2+} mobilization, Ca^{2+}/PL dependent PKC activity, and actin polymerization. This work primarily provided evidence that helped to clarify the molecular mechanism of the anti-platelet actions of DQD. Water-soluble quercetin derivatives can hence be developed as anti-thrombotic drugs.

REFERENCES

1 Beretz A, Stierle A, Anton R, Cazenave JP. Role of cyclic AMP in the inhibition of human platelet aggregation by quercetin, a flavonoid that potentiates the effect of prostacyclin. Biochem Pharmacol 1982; 31: 3597 - 600.
2 Gryglewski RJ, Korbut R, Robak J, Swies J. On the mechanism of antithrombotic action of flavonoids. Biochem Pharmacol 1987; 36: 317 - 22.
3 Rao GH, Kishore NP, White JG. Differential effects of putative inhibitors on cytosolic and membrane associated platelet lipoxygenase. Prostaglandins Leukotrienes Med 1987; 26: 281 - 90.
4 Fischer TH, Campbell KP, White GC. An investigation of functional similarities between the sarcoplasmic reticulum and platelet calcium-dependent adenosinetriphosphatases with the inhibitors quercetin and calmidazolium. Biochemistry 1987; 26: 8024 - 30.
5 Ogawara H, Akiyama T, Watanabe S, Ito N, Kobori M, Seoda Y. Inhibition of tyrosine protein kinase activity by synthetic isoflavones and flavones. J Antibiot (Tokyo) 1989; 42: 340 - 3.
6 She J, Mo LE, Kang TB, Song ZJ, Liang NC. Preparation of water-soluble quercetin derivatives and their biological activities. Chin J Med Chem 1998; 8: 287 - 89.
7 She J, Mo LE, Liang NC. Effects of temperature on preparation of quercetin sulphates. Chin J Mod Appl Pharm 1998; 15: 22 - 3.
8 She J, Mo LE, Liang NC. Study on effect of fuming sulfuric acid and quercetin. Chem Res Appl 1999; 11: 206 - 7.
9 Liu W, Song ZJ, Liang NC, She J, Mo LE. Inhibitory effects of sodium quercetin monosulfate on pig platelet aggregation induced by thrombin. Acta Pharmacol Sin 1999; 20: 623 - 26.
10 Jennings LK, Fox JEB, Edwards HH, Phillips DR. Changes in the cytoskeletal structure of human platelets following thrombin activation. J Biol Chem 1981; 256: 6927 - 32.
11 Rink TJ. Cytosolic calcium in platelet activation. Experientia 1988; 44: 97 - 100.
12 Walker TR, Watson SP. Synergy between Ca^{2+} and protein kinase C is the major factor in determining the level of secretion from human platelets. Biochem J 1993; 289: 277 - 82.
13 Phillips DR, Jennings LK, Edwards HH. Identification of membrane proteins mediating the interaction of human platelets. J Cell Biol 1980; 86: 77 - 86.
14 Kang TB, Liang NC. Studies on the inhibitory effects of quercetin on the growth of HL-60 leukemia cells. Biochem Pharmacol 1997; 54: 1013 - 18.
15 Agullo G, Gamer-Payrastre L, Manenti S, Viala C, Remesy C, Chap H and Payrastre B. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochem Pharmacol 1997; 53: 1649 - 57.

槲皮素-7,4′-二硫酸酯对凝血酶诱导猪血小板聚集的抑制作用及其机制[1]

刘 文，梁念慈[2]（广东医学院生物化学和分子生物学研究所，湛江 524023，中国）

关键词 血小板；黄酮类；槲皮素；血小板聚集；钙；蛋白激酶 C；肌动蛋白类

目的：研究人工半合成槲皮素—槲皮素-7,4′-二硫酸酯二钠（disodium quercetin-7,4′-disulfate，DQD）对凝血酶（500 U·L^{-1}）诱导猪血小板聚集的抑制作用及其机制。**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（PKC）酶液一起保温的方法测定 Ca^{2+}/PL 依赖的 PKC 活性。用 SDS-PAGE 分离骨架蛋白。**结果**：DQD 对凝血酶诱导的血小板聚集有抑制作用，当 DQD 的浓度为 100，200 和 400 μmol/L 时，抑制率分别为 77 %、86 %和 82 %。DQD（10－80 μmol/L）抑制凝血酶诱导的血小板胞外钙内流；DQD 对凝血酶诱导的血小板胞内钙动员也有抑制作用。DQD（10－160 μmol/L）抑制血小板胞浆 Ca^{2+}/PL 依赖的 PKC，但 DQD 不影响胞膜 PKC。DQD（20－200 μmol/L）对凝血酶诱导的血小板肌动蛋白聚合有较强的抑制作用。**结论**：DQD 对凝血酶诱导的猪血小板聚集有抑制作用，其分子作用机制是由于抑制血小板外钙内流、内钙动员、Ca^{2+}/PL 依赖的 PKC 和肌动蛋白聚合。

（责任编辑 吕 静）