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Research Article

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Anti-platelet aggregation mechanisms of quercetin fatty acid ester

Xiao Lan Wang and Yu Duan*

School of Pharmaceutical and Biological Sciences, Weifang Medical University, Weifang, Shandong Province, China

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ABSTRACT

The mechanisms of quercetin-3-O-acetate (QA) and quercetin-3-O-propionate (QP) on anti-platelet aggregation were researched. The results revealed that QA and QP significantly increased the generation of cAMP and decreased free calcium concentration in platelet. But there was no significant impact on cGMP level. The mechanisms of QA and QP anti-platelet aggregation maybe related to they could increase cAMP content, and inhibit free calcium concentration promotion in platelets.

Keywords: quercetin fatty acid ester; anti-platelet aggregation mechanisms; cAMP and cGMP content; calcium concentration

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INTRODUCTION

Quercetin (3, 3', 4', 5, 7-Pentahydroxyflavone, Fig. 1) is an important type of flavonoid from plant. Some researches demonstrated that quercetin could delay thrombus formation in diabetes and suggest a potential clinical role in anti-platelet therapy [1]. Previous studies showed that quercetin and its derivatives had obviously inhibitory effect on the platelet aggregation induced by ADP, AA, PAF, collagen and thrombin [2]. That may be due to quercetin could inhibit platelet calcium influx thereby inhibited platelet activation [3]. But there was no quercetin medicament used for antiplatelet treatment in clinical. This was mainly because the quercetin insoluble in water, difficult to absorption and had low bioavailability [4]. In order to exploit clinical applied quercetin derivatives, our research group synthesized series of quercetin-3-O- aliphatic esters and screened the anti-platelet activity of them. The results revealed that QA (Fig. 2) and QP (Fig. 3) had significant anti-platelet activity and better solubility than quercetin. But the mechanisms of them to anti-platelet aggregation had not been reported in any report. In this paper the level of cAMP, cGMP and free calcium in platelet were researched to reveal the mechanisms of quercetin-3-O- aliphatic ester anti-platelet activity.

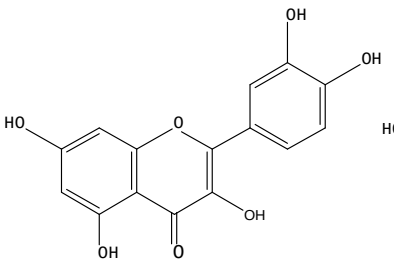

Fig. 1: chemical structure of quercetin

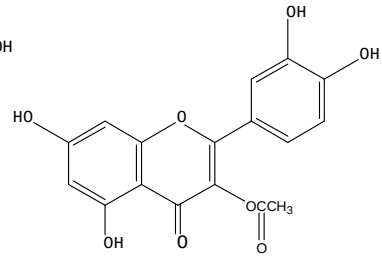

Fig. 2: chemical structure of quercetin-3-O-acetate

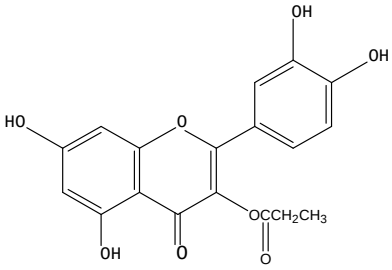

Fig. 3: chemical structure of quercetin-3-O-propionate

EXPERIMENTAL SECTION

Materials and Instruments

QA and QP were synthesized by Pharmaceutical Laboratory, School of Pharmaceutical Sciences, Weifang Medical University.

cAMP and cGMP EIA kits were purchased from Amersham (Sweden). Flua-2/AM was purchased from Biotium, Inc (Hayward, CA, USA). THR and DMSO were purchased from Sigma-Aldrich (St. Louis. Mo., USA). EGTA, HEPES, TritonX-100 and Albumin Bovine were purchased from Amresco LLC (Solon, OH, USA). The other regents were analytical reagents. The water used in the experiment was triple-distilled water. The HEPES buffer solution with calcium-free included: NaCl 135 mmol/L, KCl 5 mmol/L, $MgSO_4$ 1mmol/L, Hepes 10 mmol/L, Glucose 10 mmol/L, pH7.40.

Japanese white rabbits, male, body weight (3.0±0.5) kg were provided by Experimental Animal Center of Weifang Medical University. The animal research plan had obtained Weifang Medical University Ethics Committee's examination and authorization.

The fluorescence analysis was carried out on the Shimadzu RF5301 (Japanese) fluorescence spectrophotometer.

The enzyme immunoassay was carried out on Thermo MK3 enzyme micro-plate reader.

Assay of cAMP and cGMP content [5]

The blood sample was collected from a male Japanese white rabbit with 3.8% sodium citrate as the anticoagulant. Platelet rich plasma (PRP) was obtained by removing erythrocytes and leukocytes on centrifugation (1000 rpm, 10 mins). Platelet poor plasma (PPP) was prepared by further centrifugation (3000 rpm, 15 mins) and platelet precipitation was obtained in the bottom of centrifuge tube. The number of platelets in the precipitation was obtained by calculating the platelet count difference between PRP and PPP. Normal saline and triple-distilled water were added to the precipitation and kept for 2 minutes to dissolve the residual erythrocytes, and then 4.5% sodium chloride was added to recovery isotonic. Then the plasma was centrifuged (3000 rpm, 15 mins) and the obtained precipitation was suspended in normal saline. The platelet suspensions (0.5 mL) were treated with different drugs or normal saline (control) and incubated for 15minutes at 37℃. The incubation was terminated by adding trichloroacetic acid (10%, w/v) to destroy the platelets and then centrifuged to obtain the supernatant. The trichloroacetic acid in the supernatant was extracted by diethylether for three times. The aqueous phase of the extract was lyophilized and cryopreservated at -30℃. cAMP and cGMP were measured by enzyme immunoassay using EIA Kit.

Measurement of cytosolic free calcium concentration [6]

The blood sample was collected from a male Japanese white rabbit with 3.8% sodium citrate as the anticoagulant. After mixing, platelet rich plasma (PRP) was obtained by removing erythrocytes and leukocytes through centrifugation (1000 rpm, 10 mins). Platelet precipitate was obtained from PRP by centrifugation (2500 rpm, 15 mins), and the precipitate was washed in HEPES-Tyrode buffer for two times. Then the platelet precipitate was suspended in HEPES-Tyrode buffer to a final concentration of 5×10^9 /L. Then the platelet suspension was loaded with Fura-2/AM (final concentration: 5μmol/L) and incubated away from the light for 45 minutes. The loaded platelet was suspended in HEPES and regulated the platelet count to 1.0×10^8/mL. The cytoactive was detected by MTT Assay and the cell livability > 95%. Then divided the platelet suspension into two groups: one added $CaCl_2$ to 1mmol/L and the other added EGTA to 1mmol/L, interacting for 10 minutes before the fluorescence intensity was determined. Fluorescence (F) in resting state of different drugs and normal saline was measured at first. Then THR (200U/L) was added to read the active state fluorescence (F_T). Finally, fluorescence minimum (F_{min}) was measured after adding 0.1% Triton X-100, then added 10mmol/L EGTA to get fluorescence maximum (F_{max}) .The concentration of Ca^{2+} can be calculated according to the following formula (K_a=224nmol/L):

$$[ca^{2+}]_i = Ka \times \frac{F - F\min}{F\max - F}$$

RESULTS AND DISCUSSION

Effect of QA and QP on the content of cAMP and cGMP in rabbit platelet

The determinations of cAMP and cGMP in platelet were performed according to enzyme-linked immunosorbnent assay. The blood sample from NS group contained (15.81±3.74) pmol cAMP and (1.18±0.30) pmol cGMP/10^7 platelets. In the concentration range of inhibiting platelet aggression in *vitro* and *vivo*, 1, 10 and 100 μmol/L QA and

QP all could increase the content of cAMP, but had no obvious impact on cGMP level in platelet. There were statistical differences compared with the NS group. The results were presented in Table 1 and Table 2.

Tab 1 Effect of QA on the formation of cAMP and cGMP in rabbit platelet. *n*=10, $\bar{X} \pm S$

Group	*c* (μmol/L)	cAMP Platelet/10^7 pieces	cGMP Platelet/10^7 pieces
NS		15.81±3.74	1.18±0.30
QA	1	18.13±4.97	1.24±0.27
	10	20.71±5.41^{*1}	1.26±0.19
	100	23.96±4.92^{*2}	1.31±0.20

Note: $^{*1}P$ <0.05, $^{*2}P$<0.01 vs NS group

Tab 2 Effect of QP on the formation of cAMP and cGMP in rabbit platelet. *n*=10, $\bar{X} \pm S$

Group	*c* (μmol/L)	cAMP Platelet/10^7 pieces	cGMP Platelet/10^7 pieces
NS		15.81±3.74	1.18±0.30
QP	1	17.25±4.01	1.28±0.29
	10	21.92±4.17^{*1}	1.20±0.32
	100	23.43±6.34^{*2}	1.29±0.30

Note: $^{*1}P$ <0.05, $^{*2}P$<0.01 vs NS group

Effect of QA and QP on $[Ca^{2+}]_i$ in rabbit platelet

The $[Ca^{2+}]_i$ was measured according to the method of Flura-2- AM fluorescent probe spectra. The results showed that the calcium concentration significantly increased when induced by THR no matter there was extracellular calcium or no (P<0.01).

After interacting with 1, 10 and 100 μmol/L QA and QP respectively, we found that they could dose-dependently inhibit the active state $[Ca^{2+}]_i$ increase, regardless there was extracellular calcium or no (P<0.05 or P<0.01). At the same time we found that QA and QP had no significant effect on the resting $[Ca^{2+}]_i$ (P>0.05). The results were presented in Table 3 and Table 4.

Tab 3 Effect of QA on cytosolic free Ca^{2+} in rabbit Platelet. *n*=10, $\bar{X} \pm S$

Group	*c* (μmol/L)	with 1 mol/L Ca^{2+}		with 1 mol/L EGTA	
		Before THR	After THR	Before THR	After THR
NS		79.46±19.23	380.78±64.29^{*4}	50.08±12.34	360.43±66.19^{*4}
QA	1	80.97±18.25	290.34±60.34^{*1}	49.56±13.43^{*3}	273.78±55.64^{*1}
	10	82.34±20.44	160.76±37.78^{*2}	52.04±14.11^{*3}	133.42±36.01^{*2}
	100	83.15±19.47	80.03±20.32^{*2}	51.45±13.87^{*3}	61.44±19.07^{*2}

Note: $^{*1}P$<0.05, $^{*2}P$<0.01, vs NS group; $^{*3}P$<0.05, vs the group before THR treatment with 1 mmol/L Ca^{2+}, $^{*4}P$<0.01, vs the group before THR treatment

Tab 4 Effect of QP on cytosolic free Ca^{2+} in rabbit Platelet. *n*=10, $\bar{X} \pm S$

Group	*c* (μmol/L)	with 1 mol/L Ca^{2+}		with 1 mol/L EGTA	
		Before THR	After THR	Before THR	After THR
NS		79.46±19.23	380.78±64.29^{*4}	50.08±12.34	360.43±66.19^{*4}
QP	1	79.85±17.02	296.46±59.34^{*1}	50.93±12.78^{*3}	298.67±54.71^{*1}
	10	82.55±19.19	175.76±40.21^{*2}	56.27±14.42^{*3}	150.36±36.24^{*2}
	100	83.47±19.64	91.14±22.58^{*2}	51.96±13.61^{*3}	77.99±24.41^{*2}

Note: $^{*1}P$<0.05, $^{*2}P$<0.01, vs NS group; $^{*3}P$<0.05, vs the group before THR treatment with 1 mmol/L Ca^{2+}, $^{*4}P$<0.01, vs the group before THR treatment

DISCUSSION

Cardiovascular disease is one of the major causes of death in the world today [7-9]. Platelet aggregation plays a significant role in the cardiovascular disease and involves complex mechanisms. cAMP and cGMP are the inhibitory second messengers in platelet and can weaken or prevent activator-induced platelet activation [10-13]. The present work demonstrated that QA and QP could increase cAMP content, but had no obvious impact on cGMP level in platelet. According to reports, cAMP in platelet plays a regulatory role on platelet function. It mainly performed in the following aspects: reducing cytoplasmic Ca^{2+} concentration, reducing the combination of platelet with THR, inhibiting the formation of DG and IP_3 mediated by PLC, leading to the phosphorylation of MLCK by cAMP-dependent protein kinase, and so on. So we can say the ability of increasing cAMP level in platelet may be the mechanism of QA and QP to inhibit platelet aggression.

The experiments also showed that in the case of presence calcium or no, QA and QP had no significant effect on the platelet resting free $[Ca^{2+}]_i$. It indicated that QA and QP did not affect the release of platelet basic state calcium. When extracellular $[Ca^{2+}]_i$ was 1mmol/L, the free $[Ca^{2+}]_i$ rise that induced by inducer was mainly caused by extracellular calcium influx. Whereas in the case of absence of extracellular calcium, the $[Ca^{2+}]_i$ rise induced by inducer mainly depended on the release of intracellular calcium. The experiment results showed that QA and QP could dose-dependently inhibited the $[Ca^{2+}]_i$ rise that induced by THR whether the calcium presence or free. It suggested that QA and QP could inhibit extracellular calcium influx and intracellular calcium release.

CONCLUSION

As we all knew, calcium as second messengers played a key role in the process of platelet activation. It was an important regulatory factor of the platelet metabolism and function. When stimulated, intracellular free calcium concentration increased mainly dependent on the receptor-mediated calcium channel opening and the calcium in the platelet pipeline system releasing [14]. According to research, cAMP could reduce the platelet calcium increase that stimulated by inducer. The way cAMP regulated Ca^{2+} was to activate calcium pump by cAMP-dependent protein kinase, then it could promote Ca^{2+} reuptake or inhibit calcium influx. Our research indicated, QA and QP could dose-dependently rise cAMP in the platelet, so we speculated QA and QP could reduce calcium concentration may be related to increase cAMP and then promote cytosolic Ca^{2+} reuptake or block calcium channel to decrease intracellular calcium influx. Now that platelet activation mainly by the following three ways: ADP, PAF and AA-TXA_2. The three ways all activated platelets by increasing Ca^{2+} concentration in platelet. Our study showed that, QA and QP could reduce calcium levels in platelets, block platelet activation common pathway, and then we can conclude that the mechanisms of QA and QP inhibiting platelet activating were closely related to its anti-calcium effect.

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