

# Effects of glycosaminoglycan from *Urechis unicinctus* on the P<sub>2</sub>Y<sub>1</sub> receptor pathway and expression of related factors in rat platelets

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**Abstract:** The aim of this study was to examine the effects of glycosaminoglycan (GAG) from *Urechis unicinctus* on the P<sub>2</sub>Y<sub>1</sub> receptor pathway and expression of related factors in rat platelets. The concentration of calcium ion (Ca<sup>2+</sup>) in rat platelets was determined by double wavelength Fura-2 fluorescence spectrophotometry, and the concentrations of inositol trisphosphate (IP<sub>3</sub>) and glycoprotein IIb/IIIa (GPIIb/IIIa) in rat platelets were measured using the enzymatic immunoassay method. The phosphorylation levels of phospholipase C (PLC), phospholipase A<sub>2</sub> (PLA<sub>2</sub>), protein kinase C (PKC), and p38 mitogen-activated protein kinase (p38MAPK) were also detected by Western blot. It was found that the GAG from *U. unicinctus* significantly reduced the Ca<sup>2+</sup> and IP<sub>3</sub> levels in rat platelets ( $p < 0.05$ ,  $p < 0.01$ ). Moreover, medium and high concentrations of GAG significantly reduced the concentration of the platelet membrane GPIIb/IIIa in rats ( $p < 0.05$ ,  $p < 0.01$ ). The phosphorylation levels of PLC, PLA<sub>2</sub>, PKC and p38MAPK in rat platelets were also inhibited by GAG and P<sub>2</sub>Y<sub>1</sub> receptor blocker MRS2179 ( $p < 0.05$ ,  $p < 0.01$ ). However, the degree of inhibition of GAG was lower than that of MRS2179. The results laid a foundation for further utilization of the glycosaminoglycan.

**Keywords:** *Urechis unicinctus*, glycosaminoglycan, P<sub>2</sub>Y<sub>1</sub> receptor pathway, western blot.

## INTRODUCTION

The incidence and mortality of thrombotic diseases continue to rise, and therefore, antithrombotic drugs are greatly important in the treatment and prevention of thrombotic diseases (Ma *et al.*, 2017). At present, the drugs used for the clinical prevention and treatment of thrombotic diseases mainly include anticoagulant, thrombolytic and antiplatelet aggregation drugs. Platelet adhesion and activation are important risk factors for thrombosis formation and development, and they are also associated with the main physiological and pathological mechanisms of ischemic diseases, such as myocardial infarction or atherosclerosis; these factors seriously threaten human health (Bigalke *et al.*, 2009). Platelets also play an essential role in hemostasis and wound healing by facilitating thrombus formation at sites of injury (Jackson *et al.*, 2016). Receptor agonists, such as collagen, thrombin and ADP, bind to their receptors and induce platelet activation through different signaling pathways, which cause calcium mobilization, granulation, and activation of the integrin  $\alpha$ IIb $\beta$ 3, leading to platelet degranulation, aggregation, and thrombosis (Han *et al.*, 2006). Platelet aggregation and thrombosis are the major pathogenic factors of cardiovascular and cerebrovascular diseases; therefore, the development of new, safe and effective antiplatelet aggregation drugs is of great significance (Shi *et al.*, 2007). Antiplatelet drugs prevent thrombosis formation by inhibiting platelet adhesion, aggregation, and degranulation (Jesus *et al.*, 2017). Although antiplatelet drugs have achieved significant

clinical efficacy, the mortality and disability rates related to their usage remain high. Therefore, finding new targets, developing new drugs and reducing the risk of bleeding are current issues that need to be addressed in the study of antiplatelet aggregation drugs.

Human platelets have three different ADP receptors: P<sub>2</sub>Y<sub>1</sub>, P<sub>2</sub>Y<sub>12</sub> and P<sub>2</sub>X<sub>1</sub>. The P<sub>2</sub>X<sub>1</sub> is a ligand-gated ion channel, and P<sub>2</sub>Y<sub>1</sub> and P<sub>2</sub>Y<sub>12</sub> are receptors coupled with two different G proteins. The P<sub>2</sub>Y<sub>1</sub> and P<sub>2</sub>Y<sub>12</sub> are the receptors for the action of ADP and are the targets of ADP receptor blockers (Jesus *et al.*, 2017).

Glycosaminoglycan (GAG) is a class of linear polyanionic long-chain molecules with carboxyl and sulfate groups consisting of repeating complex units of hexuronic acid and hexosamine; they widely exist on the surfaces of animal cells and in the extra cellular matrix (Wang *et al.*, 2010). As an important biological macromolecule in the body, glycosaminoglycan participates in important biological processes, such as cell proliferation, differentiation, and immune regulation, through their combination with various enzymes, growth factors, and cell adhesion factors *in vivo* (Mourao *et al.*, 1998). Glycosaminoglycan from sea cucumber exhibits significant anticoagulant and antithrombotic activities (Yuan *et al.*, 2014). Some scholars have extracted and purified GAG from *U. unicinctus* and investigated their composition structure, anti-clotting and thrombolytic activities (Cui *et al.*, 2015; Yuan *et al.*, 2014; Chu *et al.*, 2010; Yang *et al.*, 2010). We found that such GAG exert an obvious antiplatelet aggregation effect (Miao *et al.*, 2018). To explore the antiplatelet aggregation mechanism

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of GAG and provide a theoretical basis for their use, we studied their effects on the  $P_2Y_1$  receptor pathway and the expression of related factors in rat platelets by using adenosine diphosphate (ADP) as inducer.

## MATERIALS AND METHODS

### Experimental materials

#### Animals

Specific pathogen-free *rattus norvegicus* (SD) rats (weighing 250±22g) were purchased from the Experimental Animal Center of Military Medical Science Academy of the Chinese People's Liberation Army and animal certificate was SCXK-(Army) 2012-0004.

#### Drug and doses

Glycosaminoglycan from *U. unicinctus* was purified by the Laboratory of Oceanic Bioactive Material Utilization of Tianjin University of Science and Technology, China. The purity was 97.32%, and the contents of sulfate, uronic acid, and amino sugar in GAG were 30.26%, 25.25%, and 7.58%, respectively. Sodium ozagrel (SO, approval number: H20052521; Shijiazhuang Siyao Co., Ltd.). Adenosine diphosphate, and calcium fluorescent probe Fura-2/AM were purchased from Sigma Company. The rat inositol trisphosphate ( $IP_3$ ) ELISA kit was purchased from the Nanjing Institute of Bioengineering. The rat platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa) ELISA kit was purchased from Shanghai Yuchun Biotechnology Co., Ltd. The antibodies of p38 mitogen-activated protein kinase (p38MAPK), p-p38MAPK, phospholipase C (PLC), p-PLC, phospholipase  $A_2$  (PLA $_2$ ), p-PLA $_2$ , protein kinase C (PKC) and p-PKC were purchased from Cell Signaling Technology (USA),  $\beta$ -actin antibody was purchased from the Nanjing Institute of Bioengineering. TRIZOL reagents were purchased from Invitrogen Company.

#### Instruments

The following instruments were used: Infinite 200 Pro multifunctional microplate reader (Tecan, Switzerland), XFA6030 automatic blood cell analyzer (Nanjing Prong Medical Equipment Co., Ltd.), DYCZ-22B vertical electrophoresis apparatus and DYCZ-24KF transfer printing electrophoresis instrument (Beijing Liuyi Instrument Factory), RF-5301 fluorescence spectrophotometer (Shimadzu Corporation), T100 MyCycler PCR instrument and ChemiDoc XRS gel imaging system (Bio-Rad, USA) and Image Quant LAS 4000 chemiluminescence imaging instrument (GE Healthcare Life Sciences).

#### Experimental methods

Fifty rats were randomly divided into five groups: the control group, the positive control group (SO), and GAG groups (high, medium, and low concentration), with 10 rats in each group. Normal saline, SO (4mg/kg) and

GAGs (4, 8, 16mg/kg) were administered through the caudal vein with a total volume of 0.5mL for each injection. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared and used to determine the calcium ion,  $IP_3$  and GPIIb/IIIa concentrations in the rat platelets.

Forty rats were then randomly divided into four groups: Resting group, control group, GAG group and MRS2179 ( $P_2Y_1$  inhibitor) group, with 10 rats in each group. The PRP was prepared and incubated for 10 min with normal saline, GAG and MRS2179, respectively. In addition to the resting group PRP, the other groups' PRP was stimulated for 5min with ADP and the stimulation was stopped with an ice bath. The PRP was then used for subsequent Western blot.

#### Preparation of PRP and PPP

After 30 min of administration, blood was collected from the femoral artery and then anticoagulated with 3.8% sodium citrate (the ratio of blood to anticoagulant was 9:1). The anticoagulant blood was centrifuged at 1500 r/min for 5min to obtain PRP. Subsequently, PRP was centrifuged at 4000 r/min for 10 min to obtain PPP.

#### Determination of intracellular calcium ion ( $[Ca^{2+}]_i$ ) concentration in rat platelets

Based on a previous method with slight modification (Yang *et al.*, 2010), platelet preparations were added into 5 $\mu$ mol/L Fura-2/AM and incubated for 40 min. Centrifuged precipitates were washed twice with and resuspended with Hepes buffer and then the platelet was adjusted to a concentration of 3 $\times 10^9$ /mL. After 5 min of stimulation by ADP, fluorescence signals were recorded using the RF-5301 fluorescence spectrofluorometer.

Fluorescence emission was determined at 510 nm, with simultaneous excitation at 340 and 380nm, changing every 0.5 s. Platelet  $[Ca^{2+}]_i$  was calculated based on the following formula:

$$[Ca^{2+}]_i = Kd \times \frac{R-R_{min}}{R_{max}-R} \times SFB$$

In the formula, Kd represents the dissociation constant of the reaction of the Fura-2/AM with  $Ca^{2+}$ , which was 224 nmol/L. R represents the ratio of fluorescence intensity (F340/F380), and  $R_{max}$  is the ratio of fluorescence intensity (F340/F380) when Fura-2 and calcium ion reached saturation by adding TritonX-100.  $R_{min}$  represents the ratio of fluorescence intensity (F340/F380), which was achieved by adding EGTA at a concentration two to three times higher than the  $Ca^{2+}$  concentration to free Fura-2. SFB is the ratio of  $R_{min}$  and  $R_{max}$  at 380 nm.

#### Determination of $IP_3$ in rat platelets

The PRP (platelet count was 3 $\times 10^9$ /mL) was stimulated for 5 min with ADP. The reaction was terminated with an ice bath, and the suspension was centrifuged (4000 r/min

for 10 min). The PMSF (protease inhibitor, avoiding the catalytic reaction of the enzyme) was added into the suspension, and the suspension was frozen and thawed for three times (-80°C to 37°C) and centrifuged (4000 r/min for 10 min). The supernatant was used to determine the IP<sub>3</sub> concentration as indicated by the manufacturer's instructions.

#### Determination of the GPIIb/IIIa concentration in rat platelets

The sample pretreatment was the same as that with Subsection 1.2.3. The supernatant was used to determine the GPIIb/IIIa concentration in accordance with the kit's instructions.

#### Western blot

The PRP (platelet count was  $3 \times 10^9$ /mL) was stimulated for 5 min with ADP. The reaction was terminated with an ice bath, and the suspension was centrifuged (4000 r/min for 10 min). The platelets were washed two times with PBS and cracked for 30 min (4 °C) by adding RIPA lysate (protease and phosphatase inhibitor were added ahead of time). The suspension was centrifuged at 5000 r/min for 5 min, and the supernatant was regarded as the total cell protein. The protein was separated by SDS-polyacrylamide gel electrophoresis (the volume of the protein sample was 10µg/lane), and the isolated protein was transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF film was placed in 5% BSA, which was rocked and closed for 1 h at room temperature and incubated overnight at 4°C with prediluted rabbit antibodies against PLC, p-PLC, PLA<sub>2</sub>, p-PLA<sub>2</sub>, p38MAPK, p-p38 MAPK, PKC and p-PKC. The film was washed for 5min, incubated for 1h with Goat anti-Rabbit IgG labeled with horseradish peroxidase at room temperature and then washed for 5min. ECL chemiluminescent liquid was used for color reaction. A chemiluminescence imager was used to detect the chemiluminescence and preserve the image. The Image Quant TL density analysis software was used to analyze the grayscale of protein bands in the image.

#### Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

## STATISTICAL ANALYSIS

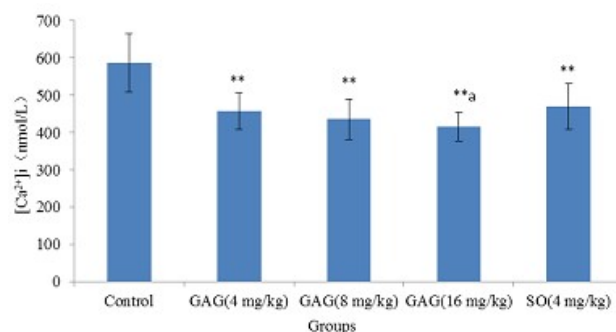
One-way analysis of variance was used for data analysis followed by SPSS 17. All data were expressed in mean ± SD.

## RESULTS

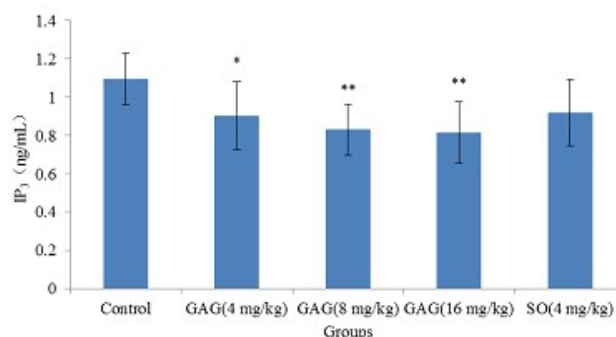
#### Effects of GAG on the Ca<sup>2+</sup> concentration in rat platelets

As shown in fig. 1, different concentrations of GAG and

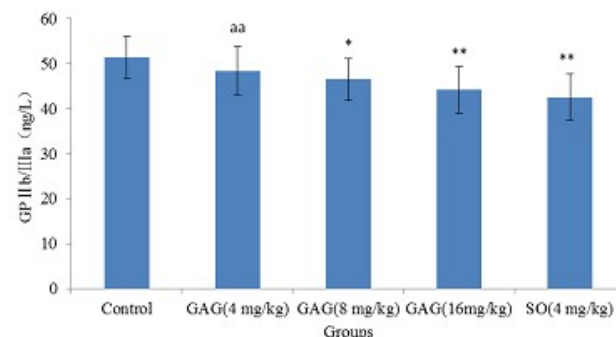
positive drugs could significantly reduce the Ca<sup>2+</sup> concentration in the platelets ( $p < 0.01$ ). The degree of reduction of the high-concentration GAG was significantly higher than that of the positive drugs ( $p < 0.05$ ). Meanwhile, the degree of reduction of the medium and low concentration GAG was obviously higher than that of the positive drugs but did not reach a significant level ( $p > 0.05$ ).



**Fig. 1:** Effects of glycosaminoglycan on the concentration of calcium ion in the rat platelets. \*\* $p < 0.01$  vs. control group, <sup>a</sup> $p < 0.05$  vs. SO group.



**Fig. 2:** Effects of glycosaminoglycan on the IP<sub>3</sub> concentration in rat platelets \* $p < 0.05$ , \*\* $p < 0.01$  vs. control group.

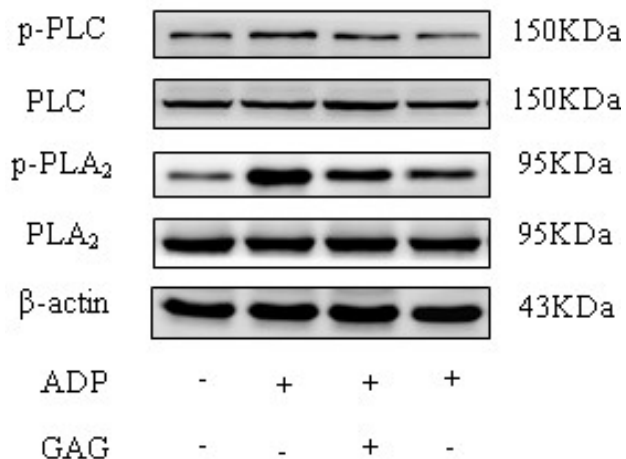


**Fig. 3:** Effects of glycosaminoglycan on the GPⅡb/Ⅲa concentration in rat platelets \* $p < 0.05$ , \*\* $p < 0.01$  vs. control group, aa $p < 0.01$  vs. SO group

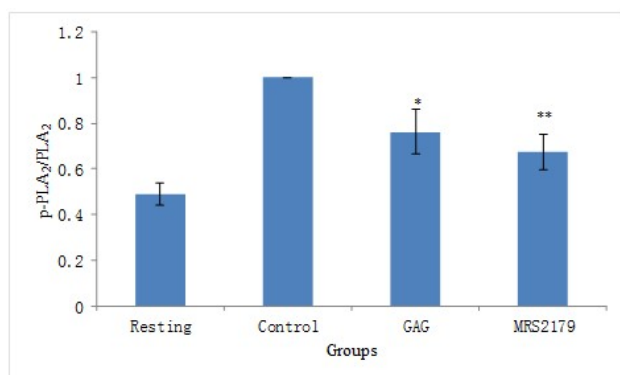
#### Effect of GAG on the IP<sub>3</sub> concentration in rat platelets

As shown in fig. 2, different concentrations of GAG significantly decreased the IP<sub>3</sub> concentration in platelets

( $p < 0.05$ ,  $p < 0.01$ ) and showed a concentration dependence. The SO also reduced the concentration of  $IP_3$  in the platelets but did not reach a significant level ( $p > 0.05$ ). We also found that the degree of reduction achieved by the GAGs for the  $IP_3$  concentration was higher than that of SO but did not reach a significant level ( $p > 0.05$ ).



**Fig. 4:** Representative panels of p-PLC, PLC, p-PLA<sub>2</sub> and PLA<sub>2</sub> proteins in rat platelets



**Fig. 5:** Effects of glycosaminoglycan on phosphorylation of PLA<sub>2</sub> in rat platelets \* $p < 0.05$ , \*\* $p < 0.01$  vs. control group

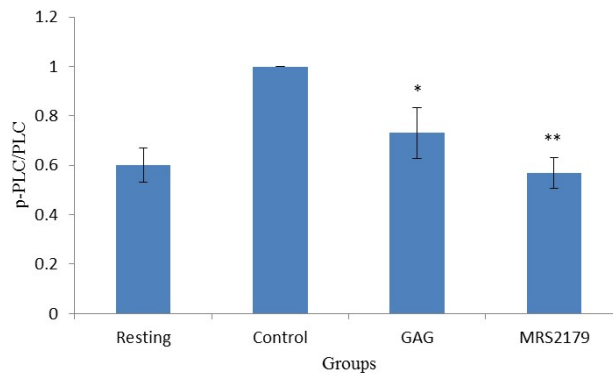
#### Effects of GAG on GPIIb/IIIa concentration in rat platelets

The result of the determination was shown in fig. 3. We found that SO and the medium and high concentrations of GAG significantly reduced the concentration of GPIIb/IIIa ( $p < 0.05$ ,  $p < 0.01$ ) in rat platelets. Low-concentration GAG also reduced the concentration of GPIIb/IIIa in rat platelets but not significantly ( $p > 0.05$ ). Meanwhile, the degree of reduction of GPIIb/IIIa concentration by SO was significantly higher than that of the medium and high concentrations of GAGs, but the difference was insignificant ( $p > 0.05$ ).

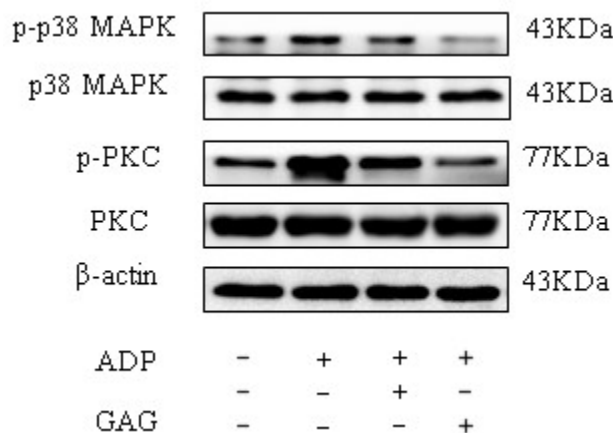
#### Effects of GAG on phosphorylation of PLA<sub>2</sub>, PLC, PKC, and p38MAPK in rat platelets

The results were shown in fig. 4-9. Glycosaminoglycan

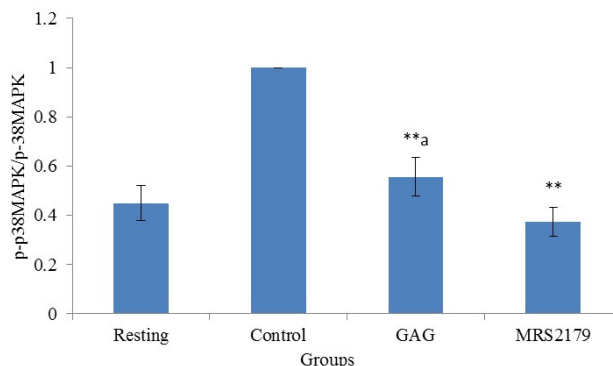
could significantly inhibit the phosphorylation of PLA<sub>2</sub>, PLC, PKC and P38MAPK in rat platelets ( $p < 0.05$ ,  $p < 0.01$ ). As a blocker of  $P_2Y_1$ , The MRS2179 could also significantly inhibit the phosphorylation of PLA<sub>2</sub>, PLC, PKC and P38MAPK in rat platelets ( $p < 0.05$ ,  $p < 0.01$ ), but the degree of inhibition was insignificant ( $p > 0.05$ ).



**Fig. 6:** Effects of glycosaminoglycan on phosphorylation of PLC in rat platelets \* $p < 0.05$ , \*\* $p < 0.01$  vs. control group.



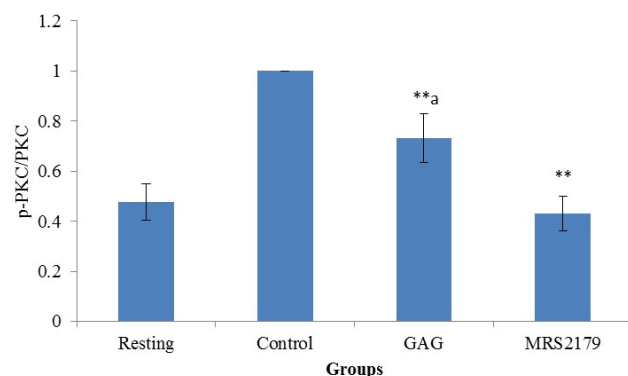
**Fig. 7:** Representative panels of p-p38MAPK, p38MAPK, p-PKC and PKC proteins in rat platelets



**Fig. 8:** Effects of glycosaminoglycan on the p38MAPK phosphorylation in rat platelets \*\* $p < 0.01$  vs. control group, <sup>a</sup> $p < 0.05$  vs. MRS2179 group

## DISCUSSION

Starting from the P<sub>2</sub>Y<sub>1</sub> receptor pathway, we studied the effects of GAG on the concentration of Ca<sup>2+</sup>, IP<sub>3</sub> and GPIIb/IIIa in rat platelets and found that GAG significantly reduced the concentrations of Ca<sup>2+</sup>, IP<sub>3</sub>, and GPIIb/IIIa in rat platelets. The P<sub>2</sub>Y<sub>1</sub> protein is the main receptor in ADP-induced platelet aggregation and is closely related to the formation of human thrombus (Yuan et al., 2016). The ADP binds to the P<sub>2</sub>Y<sub>1</sub> receptor and activates P<sub>2</sub>Y<sub>1</sub>; meanwhile, the P<sub>2</sub>Y<sub>1</sub> is coupled with the Gq protein, the Gq activates PLC and PLC hydrolyzes the phospholipid inositol to produce IP<sub>3</sub> and DAG, which promote the increase of intracellular Ca<sup>2+</sup> concentration (Mukherjee et al., 1992; Pei and Han, 2004). The binding of the platelet membrane GPIIb/IIIa with adhesion proteins, such as fibrinogen, is a necessary and final common pathway that causes platelet aggregation (Ren et al., 2005).



**Fig. 9:** Effects of glycosaminoglycan on the PKC phosphorylation in rat platelets <sup>\*\*</sup> $p < 0.01$  vs. control group, <sup>a</sup> $p < 0.05$  vs. MRS2179 group

We also estimated effects of GAG on the phosphorylation levels of PLC, PLA<sub>2</sub>, PKC and p38MAPK. The GAG significantly inhibited the phosphorylation levels of PLC, PLA<sub>2</sub>, PKC and p38MAPK. The action of activated PKC on the integrin protein  $\alpha$ IIb $\beta$ 3 promotes the opening of ligand binding sites, which is beneficial to platelet aggregation. At the same time, the PLA<sub>2</sub> was induced to activate by the increase in intracellular Ca<sup>2+</sup> concentration and was phosphorylated by p38MAPK, then releases arachidonic acid (AA) and produces TXA<sub>2</sub>, which could promote platelet aggregation (Mukherjee et al., 1992; Hechler et al., 1998; Pei and Han, 2004).

We also evaluated effects of MRS2179 on the phosphorylation levels of PLC, PLA<sub>2</sub>, PKC, and p38MAPK. In our assay, the MRS2179 could also significantly inhibit the phosphorylation levels of the four enzymes, and the inhibition of the MRS2179 was higher than that of GAG. It is reported that the MRS complex is a kind of selective competitive blocker of the P<sub>2</sub>Y<sub>1</sub> receptor, and its representative drug is MRS2179, which possesses

a strong antiplatelet aggregation effect *in vivo* and *in vitro*, the MRS2179 can significantly reduce the platelet aggregation rate induced by ADP, the expression of the P selection protein, and the activation of  $\alpha$ IIb $\beta$ 3, and inhibit the adhesion of platelets to mononuclear cells and neutrophils *in vitro* (Jesus et al., 2017; Labarthe et al., 2012).

## CONCLUSION

These findings revealed that glycosaminoglycan down regulated the concentration of Ca<sup>2+</sup>, IP<sub>3</sub> and GPIIb/IIIa in rat platelets, and also down regulated the phosphorylation levels of PLC, PLA<sub>2</sub>, PKC and p38MAPK. The MRS2179 down regulated the phosphorylation levels of PLC, PLA<sub>2</sub>, PKC and p38MAPK. It is presumed that glycosaminoglycan from *U. uncinatus* may inhibit the ADP-induced platelet aggregation in rats through the P<sub>2</sub>Y<sub>1</sub> receptor signaling pathway.

## ACKNOWLEDGEMENT

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