Ligustrazine suppresses platelet aggregation through inhibiting the activities of calcium sensors

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Ligustrazine is widely used for the treatment of cardiovascular diseases in traditional Chinese medication. It has been reported that Ligustrazine decreases the concentration of intracellular calcium ions (Ca^{2+}); however, the underlying mechanism remains unknown. In the present study, the effect of Ligustrazine on adenosine diphosphate (ADP)-induced platelet aggregation was evaluated using a turbidimetric approach. The changes in concentration of intracellular Ca^{2+} stimulated by ADP was measured using fluo-4, a fluorescent Ca^{2+} indicator dye. The mRNA expression of stromal interaction molecule l (STIM1) and Orai1, calcium sensor, was determined using real-time PCR. In addition, the protein expression of STIM1, Orai1, and serum/glucocorticoid-regulated protein kinase 1 (SGK1) was determined using Western blot analysis. The data demonstrated that Ligustrazine significantly suppressed platelet aggregation in a dose-dependent manner and reduced the concentration of intracellular Ca^{2+} triggered by ADP. Our data showed that Ligustrazine treatment inhibited the expression of STIM1 and Orai1 induced by ADP at both mRNA and protein levels, and suppressed the protein expression of SGK1. Taken together, our data indicated that Ligustrazine suppressed platelet aggregation by partly inhibiting the activities of calcium sensors, thereby suggesting that Ligustrazine may be a promising candidate for the treatment of platelet aggregation.

Keywords: Ca^{2+} release-activated Ca^{2+} channel. Intracellular calcium concentration. Orai1. Platelet activation. Stromal interaction molecule l (STIM1). Serum/glucocorticoid-regulated protein kinase 1 (SGK1).

INTRODUCTION

Platelet aggregation is vital in the aftermath of vascular injury and thrombosis (Signarvic *et al*., 2010). Accordingly, anti-platelet therapy is often used to prevent and treat cardiovascular diseases (Russo *et al*., 2017; Barrett *et al*., 2019). However, common clinical anti-platelet drugs, including aspirin and clopidogrel, pose a potential risk of causing hemorrhage, and drug resistance may occur. The elevation of intracellular calcium ions (Ca^{2+}) is a significant marker of platelet aggregation, which in turn facilitates the aggregation and thrombosis of platelets (Barendrecht *et al*., 2017; Schieber *et al*., 2017).

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Accordingly, inhibiting the influx of the level of intra-platelet Ca^{2+} provides an alternative method of preventing platelet aggregation and thrombosis.

There are two major Ca^{2+} sources that contribute to the increase in cytoplasmic Ca^{2+} in platelets. The first involves intracellular Ca^{2+} storage and the second invovles an influx of extracellular Ca^{2+} reserves (Davlouros *et al*., 2016; Noy *et al*., 2018). Upon platelet aggregation, inositol trisphosphate (IP_3) mediates the release of Ca^{2+} from the Ca^{2+} storage in platelets, which results in an elevated intracellular Ca^{2+} concentration. Alternatively, reduction of the intracellular Ca^{2+} concentration activates the Ca^{2+} sensor, stromal interaction molecule l (STIM1), which connects the cell membrane and binds to the membrane-bound Orai1 to form a STIM1-Orai1 complex. This STIM1-and-Orai1 complex co-regulates Ca^{2+} release-activated Ca^{2+} (CRAC) channels, which enables an influx

of extracellular Ca^{2+} across the membrane and initiates the aggregation of platelets (Huang *et al*., 2018; Frischauf *et al*., 2016; Bodnar *et al*., 2017; Wu, 2012). STIM1 and Orai1 proteins, therefore, play a crucial role in regulating extracellular Ca^{2+}.

STIM1 and Orai1 are regulated by the serum/glucocorticoid-regulated protein kinase 1 (SGK1) (Lang, Eylenstein, Shumilina, 2012). Acting upstream of the STIM1-Orai1 pathway, SGK1 is considered a potent regulator of platelet function (Lopez, Salido, Rosado, 2017; Lang *et al*., 2013). Ligustrazine, which chemical name is tetramethylpyrazine (TMPZ), is an active constituent of *Rhizoma ligusticum* that is frequently used in traditional Chinese medicine to prevent the onset or delay the progression of cardiovascular diseases (Guo, Liu, Shi, 2016; Qian *et al*., 2014; Zhao, Liu, Chen, 2016). It has previously been demonstrated that treatment with Ligustrazine inhibits both the influx of extracellular matrix Ca^{2+} and the release of Ca^{2+} from intracellularly stored Ca^{2+} in smooth muscle cells (Zhang *et al*., 2018) or in colonic epithelial cells (Zhu *et al*., 2006). Data have shown that treatment with Ligustrazine inhibits Akt phosphorylation, TXA2 formation, and Ca^{2+} mobilization (Li *et al*., 2018). Furthermore, Ligustrazine inhibits platelet aggregation *in vitro* (Yen *et al*., 1997); however, the underlying mechanisms involved remain to be elucidated.

Since Ligustrazine reduces the intra-platelet Ca^{2+} concentration, we hypothesized that this modulation may be through the role of Ligustrazine on calcium sensors, STIM1 and Orai1. In the present study, we evaluated the effects of Ligustrazine on the platelet aggregation rate and intracellular Ca^{2+} levels induced by adenosine diphosphate (ADP). Subsequently, mRNA levels of STIM1 and Orai1 were determined using real-time PCR and the protein expression of STIM1, Orai1, and SGK1 was determined using Western blot analysis.

MATERIAL AND METHODS

Reagents

Ligustrazine hydrochloride was purchased from Pingguang Pharmaceutical (Hefei, China). ADP was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-4 was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The BCA protein assay kit, SDS-PAGE gel preparation kit, RIPA lysis buffer, phenylmethylsulfonyl fluoride (PMSF), and skimmed milk were purchased from Genshare Biological (Xi'an, China). Anti-rat Orai1 and anti-rat STIM1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rat SGK1 antibody was purchased from Signalway Antibody LLC (College Park, MD, USA). Primary antibodies directed against β-actin and GAPDH as well as HRP-goat anti rabbit or HRP-goat anti mouse IgG antibodies were purchased from Ray Antibody Biotech (Beijing, China).

Animals

Male Sprague-Dawley (SD) rats, 2 months of age, weighing 250 ± 30 g, were purchased from the Animal Experiment Center of Henan Province (Zhengzhou, China). Animals were housed in individually-ventilated cages at an ambient temperature of 22 ± 2°C and a 12 h light/12 h dark cycle at the Experimental Animal Center at the Second Clinical Medical College of Henan University of Chinese Medicine (Zhengzhou, China). All animal procedures were conducted in accordance with the guidelines of the ethics committee at the Second Clinical Medical College of Henan University of Chinese Medicine (Zhengzhou, China), approval number: PZ-HNSZYY-2018-011.

Platelet aggregation

SD rats were anesthetized with 1% sodium pentobarbital by intraperitoneal injection (45 mg/kg). Nine parts of blood collected from the abdominal aorta were mixed with one part of 3.8% sodium citrate. The mixture was centrifuged at 120 g for 15 min at room temperature (RT) to obtain platelet-rich plasma (PRP). PRP was centrifuged at 600 g for 5 min to obtain platelet-poor plasma (PPP). The platelet concentration was adjusted with PBS to 3.0×10^8 cells/mL and incubated with 0.5, 1, or 2 mM Ligustrazine for 5 min at 37°C. As a control, an equal volume saline and 200 μL PPP

was run in parallel. The platelet aggregation rate was determined by an AggRAM platelet aggregation analyzer (Helena, Beaumont, TX, USA) in the presence of 20 μM ADP and with constant agitation at 600 rpm. The platelet aggregation rate in 5 min was measured using a turbidimetric method (Dong *et al.*, 2020). Furthermore, the effect of Ligustrazine on the platelet aggregation rate after 5, 10, and 20 min of incubation was assessed.

Measurement of intra-platelet Ca²⁺ concentration

To determine the changes in intro-platelet Ca^{2+} concentration, PRP was centrifuged at 600 g for 5 min at RT. Next, the platelets were washed twice with a calcium-free buffer (140 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 2 mM D-Glucose, 10 mM HEPES, pH 7.4) and adjusted to a concentration of 3×10^8 cells/mL. Subsequently, platelets were incubated with a fluo-4 (5 μM) Ca^{2+} indicator at 37°C for 30 min in the dark and centrifuged at 600 g for 5 min at RT. Then, platelets were washed twice and adjusted with calcium-free buffer to a concentration of 3×10^8 cells/mL. The platelet suspension was incubated with 0.5, 1 or 2 mM Ligustrazine (or an equal volume of calcium-free buffer for controls) at 37°C for 5 min. After addition of 20 μM ADP, samples were visualized under an inverted fluorescence microscope (Nikon, Kawasaki, Japan) during excitation at 494 nm and emission at 516 nm. Images of the fluorescence

emitted were obtained with a 100x objective lens (NA 1.49 oil, Nikon, Japan) and recorded with an EMCCD camera (Andor, Belfast, UK). About 10–15 cells were observed in a microscopic field. Fluorescence micrographs were analyzed with Andor iQ2 image software (Andor, Belfast, UK) and the changes in $[Ca^{2+}]i$, referred to as Ca^{2+} responses, were quantified by calculating the areas under the response curve recorded in each image, which were expressed as the integrated amplitude of Ca^{2+} responses.

Real-time PCR

Total RNA was extracted from platelets and reverse transcribed into cDNA using PrimeScript RT and gDNA Eraser kits (TaKaRa, Beijing, China). Primers for Orai1 and STIM1 were synthesized by Sangon Biotech (Shanghai, China) (Table I). The PCR was performed as follows: pre-denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, and amplification at 57°C for 30 s. The reaction was carried out by a real-time PCR system (Bio-Rad, Hercules, CA, USA). Ct values were analyzed using the relative quantitation method, wherein $2^{-\Delta\Delta Ct}$ was calculated accordingly: $\Delta\Delta Ct = $ (Ct of the target gene in the experimental group – Ct of the internal reference gene in the experimental group) – (Ct of the target gene in the control group – Ct of the internal reference gene in the control group).

TABLE I - The primer list of Orai1 and STIM1 genes

Gens	Primer	Product length (bp)
Orai1	F: 5'-CCATAAGACGGACCGACAGT-3' R: 5'-GGGAAGGTGAGGACTTAGGC-3'	136
STIM1	F: 5'-AGCTCCTGGTATGCTCCTGA-3' R: 5'-TATTTTCTCAGCCCCCTCCT-3'	135
GAPDH	F: 5'-ATGACTCTACCCACGGAAG-3' R: 5'-GGAAGATGGTGATGGGTTTC-3'	287

Western blot analysis

Total proteins were extracted and the protein concentration was determined using the BCA protein kit (Beyotime Biotechnology, Shanghai, China). Protein

samples were adjusted to a standard protein concentration and mixed with 5×SDS buffer. Next, for each sample, 40 μg of protein was loaded into each well of a 10% SDS-PAGE 5% condensed gel, and separated by applying 90 V for 40 min, then 120 V until complete resolution

was achieved. Proteins were transferred onto PVDF membranes for 40 min using a semi-wet method. PVDF membranes were blocked for 2 hrs at RT and incubated with primary antibodies directed against STIM1, Orai1, or SGK1 (1:1500 dilution) for 1 hr at RT, followed by incubation with a HRP-goat anti-rabbit IgG antibody for 1 h at RT. Target proteins were visualized using an enhanced chemiluminescence assay, and imaged using a GelDoc XR system (Bio-Rad, Hercules, CA, USA).

STATISTICAL ANALYSIS

Data were analyzed using one-way ANOVA for multiple comparisons or using Student's t-test for the difference between two groups, and presented as the mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Ligustrazine inhibits platelet aggregation in vitro

During aggregation, platelets release the endogenous agonist ADP, which binds to the ADP receptor on the platelet membrane, and thereby further promotes platelet activation (Dudzinska *et al.*, 2016). Accordingly, in this study, we used ADP to induce platelet aggregation. The results showed that the platelet aggregation rate in the presence of 20 µM ADP was $76 \pm 6\%$ (Figure 1A). Following treatment with 0.5, 1, or 2 mM Ligustrazine, the platelet aggregation rate was significantly reduced in a dose-dependent manner (Figure 1A). We also studied the influence of Ligustrazine on prolonging the exposure on the platelet aggregation rate. Together, the data demonstrated that the platelet aggregation rate increased with a longer Ligustrazine treatment time (Figure 1B). Thus, these findings suggested that the inhibitory effect of Ligustrazine on platelet aggregation reduces with time.
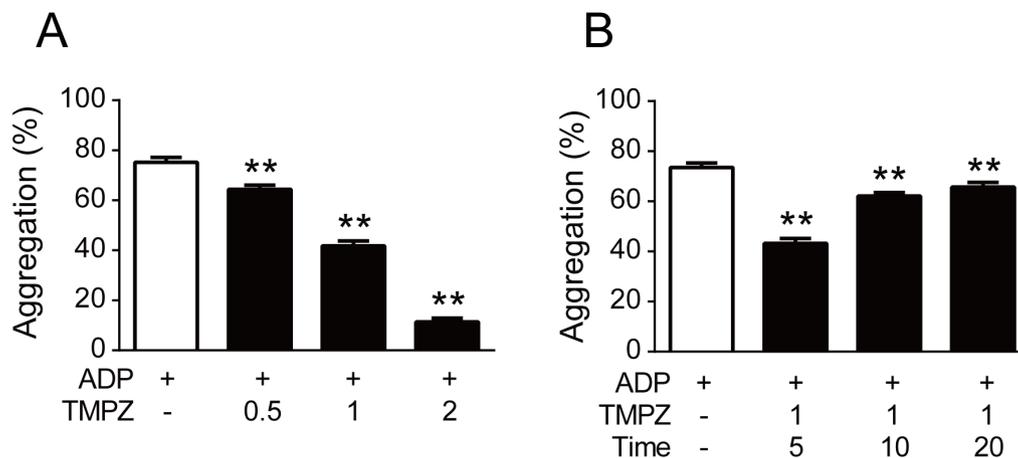
Figure 1 - Ligustrazine inhibits ADP-induced platelet aggregation *in vitro*. A: Effect of 0, 0.5, 1, and 2 mM Ligustrazine on the platelet aggregation rate. B: Effect of 5, 10, and 20 min Ligustrazine treatment periods on the platelet aggregation rate. Data are expressed as the mean ± SD. **$p<0.01$ vs control (n = 5).

Ligustrazine attenuates changes in the concentration of the intra-platelet Ca^{2+}

Elevation of the intra-platelet Ca^{2+} concentration aggravates platelet aggregation (Geue *et al.*, 2017).

Thus, changes in the intra-platelet Ca^{2+} concentration could accurately reflect the state of platelet aggregation. After washing, fluo-4-loaded platelets were visualized for the intensity of intracellular Ca^{2+} using fluorescence microscopy. ADP remarkably increased the concentration

of intra-platelet Ca^{2+}, whereas the intensity of intracellular Ca^{2+} fluorescence in platelets treated with 0.5, 1, or 2 mM Ligustrazine was lower compared to that in control platelets that were treated with saline (Figure 2). Taken together, these results indicated that Ligustrazine decreased intracellular Ca^{2+} levels in a dose-dependent manner.
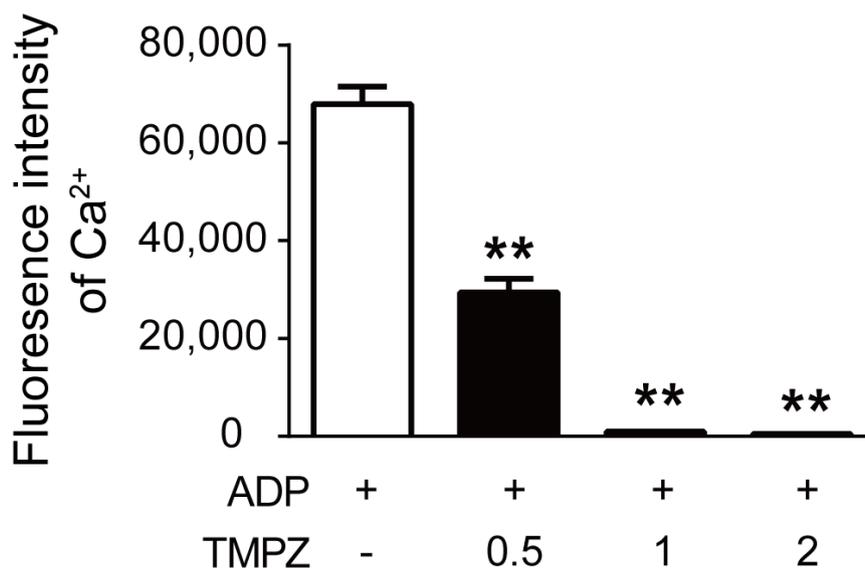

Figure 2 - Ligustrazine attenuates the intra-platelet Ca^{2+} concentration. Platelets (3×10^8 cells/mL) were labeled with fluo-4, and incubated with indicated concentrations of Ligustrazine (0.5, 1, and 2 mM) for 5 min at 37°C, followed by addition of ADP (20 µM) and real-time recording of Ca^{2+} fluorescence intensity in platelets. Data are expressed as the mean ± SD. **$p<0.01$ vs control (n = 5).

Ligustrazine reduces the mRNA expression of STIM1 and Orai1

Ligustrazine attenuated the increase in intra-platelet Ca^{2+} concentration induced by ADP (Figure 2). Therefore, we hypothesized that Ligustrazine could modulate the expression of STIM1 and Orai1 since they play a key role in regulating the Ca^{2+} concentration. We measured the expression of STIM1 and Orai1 at the mRNA level using real-time PCR, and the results showed that the mRNA expression of STIM1 and Orai1 was significantly upregulated in platelets stimulated with ADP relative to platelets that were not exposed to ADP ($P < 0.01$; Figure 3). In addition, the mRNA levels of STIM1 and Orai1 were not significantly different between platelets treated with 0.5 mM and 1 mM Ligustrazine ($P = 0.1411$ and $P = 0.069697$, respectively). In contrast, treatment with 2 mM Ligustrazine significantly reduced the mRNA expression of both STIM1 and Orai1 when compared with the positive control group that was induced with ADP ($P = 0.0462$ and $P = 0.0266$, respectively). Thus, these data suggested that Ligustrazine, when used at a concentration of 2 mM, inhibited the mRNA expression of STIM1 and Orai1.
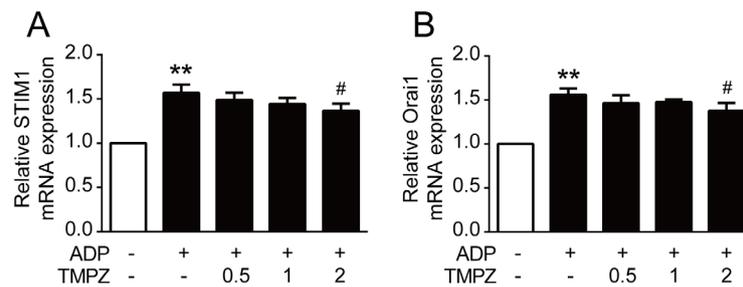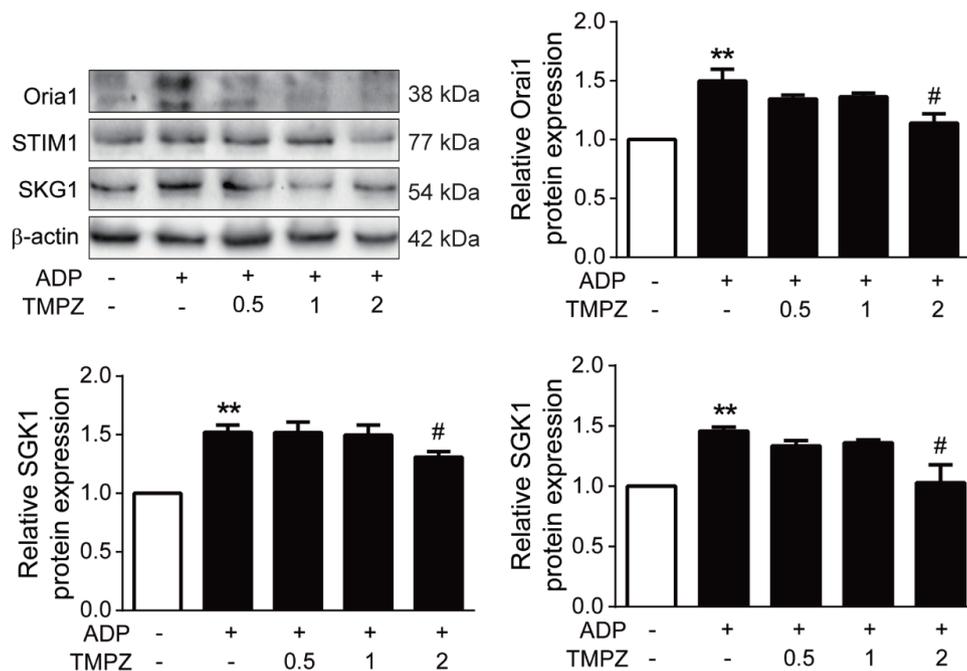
Figure 3 - Ligustrazine reduces the expression of STIM1 and Orai1 at the mRNA level. The mRNA levels of Orai1, STIM1 and GAPDH were determined using real-time PCR, and the ratios of Orai1 and STIM1 to GAPDH were calculated. Data are expressed as the mean ± SD. **$p<0.01$ vs control or #$p<0.05$ vs group with 20 μM ADP only (n = 5).

Ligustrazine suppresses the protein expression of STIM1, Orai1, and SGK1

To test the hypothesis that proteins associated with CRAC pathway modulate platelet aggregation through regulating the intra-platelet Ca^{2+} concentration, we next assessed the effects of Ligustrazine on the protein expression of STIM1 and Orai1. The data showed that ADP significantly upregulated the protein expression of STIM1 and Orai1 when compared to negative control platelets that were not exposed to ADP ($P < 0.01$;

Figure 4). Furthermore, when compared with positive control platelets induced with ADP, treatment with 2 mM Ligustrazine significantly reduced the protein expression of STIM1 and Orai1 ($P = 0.0394$ and $P = 0.0263$, respectively; $P < 0.05$), which was consistent with the inhibition in mRNA expression of STIM1 and Orai1. Similarly, our data demonstrated that 2 mM Ligustrazine decreased the protein expression of SGK1 (Figure 4). Taken together, our data suggested that Ligustrazine inhibited the protein activities of STIM1, Orai1, and SGK1.

Figure 4 - Ligustrazine suppresses the protein expression of STIM1, Orai1, and SGK1. Protein levels were expressed as a ratio of the target proteins to β-actin. Data are expressed as the mean ± SD. **$p<0.01$ vs control or #$p<0.05$ vs group with 20 μM ADP only (n = 5).

DISCUSSION

Ligustrazine is an alkaloid that is extracted from the traditional Chinese herb, *Rhizoma ligusticum* (Wu *et al*., 2019). Ligustrazine has been used for almost 30 years to treat cardiovascular diseases because of its ability to protect the vascular endothelium, inhibit platelet aggregation, and negate oxidative stress (Guo *et al*., 2016; Zhao *et al*., 2016). Regarding the anti-platelet activity of Ligustrazine, various doses of Ligustrazine were used in previous studies. For example, Sheu et al. reported that Ligustrazine (0.5-1.5 mM) dose-dependently inhibited platelet aggregation by a variety of agonists (Yen *et al*., 1997). In the present study, we applied different doses of Ligustrazine, and used a dose that was similar to the dose published by Sheu et al. (Yen *et al*., 1997). The results demonstrated that Ligustrazine inhibited the ADP-induced concentration of Ca^{2+} in platelets in a dose-dependent manner, which was consistent with the data reported previously (Volz *et al*., 2020; Wang *et al*., 2016; Chen *et al*., 2011). Although it has been reported that Ligustrazine has some effects on calcium channels (Jin *et al*., 2019), reports on the effect of Ligustrazine on calcium channels in platelets are limited.

A decrease in Ca^{2+} concentration inside the dense tubular system (DTS), which is where calcium is stored in platelets, triggers STIM1 on the DTS membrane to dimerize with Orai1 on the cell membrane, which leads to opening of the CRAC channel and a massive influx of Ca^{2+} through the cell membrane from the extracellular matrix, thereby further aggravating platelet aggregation (Derler, Romanin, 2020; Vaeth, Kahlfuss, Feske, 2020; Dolan, Diamond, 2014). Upregulated protein expression of the STIM1 and Orai1 CRAC pathway enhance the influx of Ca^{2+}, whereas downregulation of these proteins decreased the intracellular Ca^{2+} concentration (Berna-Erro *et al*., 2016). Our results showed that treatment with 2 mM Ligustrazine reduced the expression of STIM1 and Orai1 in the platelets following ADP induction at both the mRNA and protein level. Thus, these findings suggested that the changes in intracellular Ca^{2+} concentration may be associated with modulated activities of the CRAC STIM1-Orai1 pathway. We also speculated that STIM1 and Orai1 may be the most important targets for Ligustrazine. In addition, other doses of Ligustrazine used at 0.5 and 1 mM did not significantly decrease the expression of STIM1 and Orai1 at either the mRNA or protein level, which suggested that an adequate dose of Ligustrazine is required to obtain the expected effect. Furthermore, the inhibitory effect of Ligustrazine on platelet aggregation reduces with time. This is presumably due to the fast metabolism and short half-life of Ligustrazine, and suggests that in the clinic, frequent dosing may be required to maintain a therapeutic concentration.

Platelets are anucleate cells and synthesize protein using a pool of mRNAs, ribosome, and regulatory small RNAs inherited from the precursor megakaryocytes (Mills, Green, Ingolia, 2017). However, the processes involved in shaping the platelet transcriptome and platelet translation remain elusive. In our study, we found that the changes in transcription and translation occur in a very short time. Therefore, we asked whether Ligustrazine reduced the intracellular Ca^{2+} concentration by acting on STIM1 and Orai1 alone. SGK1 is a regulatory protein that is upstream of STIM1 and Orai1 in the CRAC pathway and regulates the activity of STIM1 and Orai1, and thereby influences the Ca^{2+} influx and the concentration of intracellular Ca^{2+} (Lang *et al*., 2018). By comparing wild-type and SGK1-deficient (SGK1^{-/-}) mice, platelets from SGK1^{-/-} mice showed a significantly reduced expression of Orai1 following ADP stimulation (Borst *et al*., 2012). In the present study, the expression of SGK1 was significantly decreased in 2 mM Ligustrazine-treated platelets when compared with untreated platelets. However, either 0.5 mM or 1 mM Ligustrazine did not have a significant inhibitory effect on the expression of SGK1. Thus, it is deduced that Ligustrazine reduces the concentration of intracellular Ca^{2+} in part due to modulating the activities of calcium sensors, STIM1, and Orai1.

CONCLUSION

Taken together, Ligustrazine inhibits platelet aggregation through regulating the activity of calcium sensors, STIM1, and Orai1, which enriches the theoretical evidence for the application of Ligustrazine as an anti-platelet drug in the prevention and treatment of cardiovascular diseases, especially by reducing the risk of thrombosis driven by platelet aggregation.

COMPETING INTERESTS

ACKNOWLEDGMENTS

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