

Antiplatelet drug ticagrelor delays gastric ulcer healing in rats

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Received April 6, 2016; Accepted March 24, 2017

DOI: 10.3892/etm.2017.4955

Abstract. Adenosine diphosphate P2Y₁₂ receptor antagonist clopidogrel is not sufficiently safe for the gastric mucosa in patients with high risk of peptic ulcer, since it may impair healing of gastric erosions. However, the safety of the novel P2Y₁₂ receptor antagonist ticagrelor in the gastric mucosa has not been elucidated to date. The present study aimed to examine whether ticagrelor delays gastric ulcer healing and to elucidate the involved mechanisms. Gastric kissing ulcers were produced in rats by luminal application of acetic acid solution, and ticagrelor was administered at dose of 10 or 20 mg/kg/day orally for 7 days. On day 8 after ulcer induction, the ulcer size, mucosal epithelial cell proliferation of the ulcer margin, expression levels of epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), and signal transduction pathways for cell proliferation and angiogenesis were measured and compared between the ticagrelor-treated and untreated model groups. The results revealed that the ulcer size was significantly greater in the ticagrelor-treated group compared with the model group, while the mucosal epithelial cell proliferation of the ulcer margin was significantly decreased in the ticagrelor-treated group. In addition, ticagrelor significantly decreased the ulcer-stimulated expression levels of EGF, VEGF, phosphorylated extracellular signal-regulated kinase (ERK), phosphorylated P38 mitogen-activated protein kinase and nuclear factor- κ B P65 at the ulcer margin ($P < 0.05$). These findings suggested that ticagrelor delayed gastric ulcer healing. Furthermore, the possible mechanisms underlying the effect of ticagrelor were associated with its functions of

attenuating the expression levels of VEGF and EGF, as well as suppressing the phosphorylation activation of ERK1/2, P38 and nuclear factor- κ B P65. Finally, the gastric epithelial cell proliferation and angiogenesis were also inhibited.

Introduction

In patients who have acute coronary syndromes with or without ST-segment elevation, current clinical practice guidelines recommend dual antiplatelet treatment with aspirin and adenosine diphosphate (ADP)-receptor P2Y₁₂ antagonists, including clopidogrel or ticagrelor (1,2). However, these antiplatelet agents have recognizable risks, in particular, gastrointestinal complications, including ulceration and related bleeding (3). The absolute increased risk per year of upper gastrointestinal events with aspirin is high (0.12%) when compared with placebo (4), and aspirin is associated with a 2- to 4-fold increase in gastrointestinal bleeding (5). Combined aspirin and clopidogrel is associated with significantly increased risk of gastrointestinal bleeding when compared with aspirin alone [risk ratio (RR), 1.86; 95% confidence interval (CI), 1.49-2.13] (4). Clopidogrel may not be a primary cause of gastroduodenal ulcers, and may impair healing of gastric erosions or small ulcerations that develop as a result of other medications or *Helicobacter pylori* infection (6).

Ticagrelor is a novel oral, reversible, direct-acting inhibitor of the ADP receptor P2Y₁₂ with a more rapid onset and pronounced platelet inhibition in comparison with clopidogrel, which is another P2Y₁₂ receptor (7). Patients with acute coronary syndrome treated with ticagrelor present reduced mortality rates from vascular causes, myocardial infarction or stroke, as opposed to those receiving clopidogrel treatment (7). Therefore, the American College of Cardiology/American Heart Association has suggested that ticagrelor should be preferred to clopidogrel for patients treated with an early invasive or ischemia-guided strategy (1). In addition, the European Society of Cardiology recommended clopidogrel in acute coronary syndrome patients who cannot receive ticagrelor (2).

It is well known that clopidogrel is not sufficiently safe for the gastric mucosa in patients with a high risk of peptic ulcer, since its anti-angiogenic effects may impair healing of gastric erosions. Ticagrelor has also been found to increase the risk of gastrointestinal/anal bleeding (RR=1.23; 95% confidence interval, 0.93-1.64) in a PLATO trial (8). However, to date, the

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Key words: cell proliferation, angiogenesis, P2Y₁₂ receptor antagonist, ulcer healing

potential impairment in healing of gastric erosions as a result of ticagrelor treatment have not been elucidated. The present study aimed to investigate whether ticagrelor delays gastric ulcer healing and to elucidate the involved mechanisms in a rat model of gastric kissing ulcers.

Materials and methods

Animals. A total of 32 male Sprague-Dawley rats (6 weeks old) weighing 180–200 g were obtained from Hubei Provincial Center for Disease Control and Prevention, and kept in a room with controlled temperature ($22\pm 3^{\circ}\text{C}$) and humidity ($50\pm 10\%$), and 12 h dark/light cycles. The rats were fed standard laboratory chow and tap water *ad libitum*, and acclimated 7 days before they were used for the study. All experimental procedures were performed under the Guidelines for the Care and Use of Laboratory Animals (Science and Technology Department, Hubei, China). The study protocol was approved by the Institutional Animal Care and Use Committee of Wuhan Puai Hospital (approval no. IACUC-105; Wuhan, China).

Induction of gastric kissing ulcers. Following acclimatization to the laboratory conditions for 7 days, experimental gastric ulcers were induced in the rats (9). Briefly, the rats were anesthetized with thioethamyl (35 mg/kg; Beijing Propbs Bio Co., Ltd., Beijing, China) by intraperitoneal injection. Next, the abdomen was incised and the stomach was exposed. The anterior and posterior stomach walls were clamped together with forceps with a round ring (inside diameter, 7 mm). A 60% (v/v) acetic acid solution (0.2 ml) was injected into the clamped lumen with a needle through the forestomach. After 45 sec, the acid was removed and the abdomen was closed (9). The animals were fed normally thereafter.

Drug treatment and measurement of gastric ulcer. One day after gastric ulcer induction, the animals were divided into three groups ($n=8$ each) as follows: Model group, low dose ticagrelor group and high dose ticagrelor group. A further 8 rats, which underwent a sham surgery with similar surgical procedure, but with instillation of distilled water instead of acetic acid, were set as the sham group. The low and high dose ticagrelor groups were orally treated with 10 or 20 mg/kg/day ticagrelor (AstraZeneca AB, Södertälje, Sweden) for 7 consecutive days, respectively. Ticagrelor was dissolved in physiological saline with 0.5% sodium carboxymethyl cellulose (CMC). The sham and model groups received 0.5% CMC in physiological saline without ticagrelor. The dosage of medicinal compounds was established according to previous study (10).

On day 8, all experimental animals were sacrificed. Each stomach was then opened along the greater curvature, and the areas (mm^2) of the ulcers were determined under a dissecting microscope (magnification, $\times 10$; Olympus Optical, Tokyo, Japan) with a square grid. The ulcer area was measured in a blinded manner.

Histological and immunohistochemical analysis. One ulcer part (1/3 of the ulcer) was excised, fixed with 4% paraformaldehyde, embedded in paraffin and serially sectioned ($\sim 6\times 2$ mm) for histological and immunohistochemical

examination. Gastric sections were stained with hematoxylin and eosin (H&E), and histological analysis was conducted using an optical microscope (BX51; Olympus, Tokyo, Japan) at a magnification of $\times 40$. Additionally, immunohistochemical analysis was performed using deparaffinized ulcer sections. The sections were immersed in freshly prepared 2% H_2O_2 at 37°C for 10 min and blocked with 5% goat serum for 10 min. Subsequently, primary antibodies against vascular endothelial growth factor (VEGF, ab53465, dilution 1:200; Abcam, Cambridge, UK), epidermal growth factor (EGF, ab77851, dilution 1:200; Abcam) or proliferation cell nuclear antigen (PCNA, ab18197, dilution 1:500; Abcam) were added and incubated at 37°C for 1 h. After washing with phosphate-buffered saline, the sections were treated with the secondary antibody conjugated with horseradish peroxidase at 37°C for 10 min according to the manufacturer's instructions of the kit (K500711; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Sections were then immersed in diaminobenzidine for 3 min. Following staining with hematoxylin for 1 min at room temperature, the sections were washed with H_2O , soaked in 1% hydrochloric acid for 3 sec and dehydrated using ethanol. A negative control group was obtained by omitting the primary antibody and the other operations were the same. Stained areas of the sections were visualized using an optical microscope at magnification of $\times 100$.

Biochemical analysis. The remainder part of the tissue ($\sim 1/3$ of the ulcer) was used to prepare 10% tissue homogenate by addition of ice-cold physiological saline for the measurement of EGF and VEGF levels using EGF (EK0954) and VEGF (EK0540) ELISA commercial kits (Boster Biological Technology, Ltd., Wuhan, China) according to the manufacturer's instructions.

Western blot analysis. Gastric ulcer tissues were homogenized and centrifuged at 4°C for 10 min ($9055.8 \times g$) to obtain the supernatants. Protein concentration was measured using bicinchoninic acid protein assay kit (P0010; Beyotime Institute of Biotechnology, Shenzhen, China), and then 20 μl proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Transferred membranes were blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20. These membranes were then probed with rabbit primary antibodies against extracellular signal-regulated kinase (ERK1/2; 4695) and its phosphorylated form (P-ERK; 4370), P38 mitogen-activated protein kinase (MAPK; 8690) and its phosphorylated form (P-P38) (4511), nuclear factor- κB (NF- κB) p65 (8242, all 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and β -actin (20536-1-AP, 1:10,000; Proteintech Group, Inc., Wuhan, China) overnight at 4°C . Subsequently, the membranes were incubated for 1 h with secondary antibodies conjugated with horseradish peroxidase (SA00001-2, 1:5,000; Proteintech Group, Inc.). Immunoblots were developed on films using the enhanced chemiluminescence technique (SuperSignal West Pico; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Quantification of bands was determined by densitometric analysis using Bio-Rad Quantity One 4.52 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

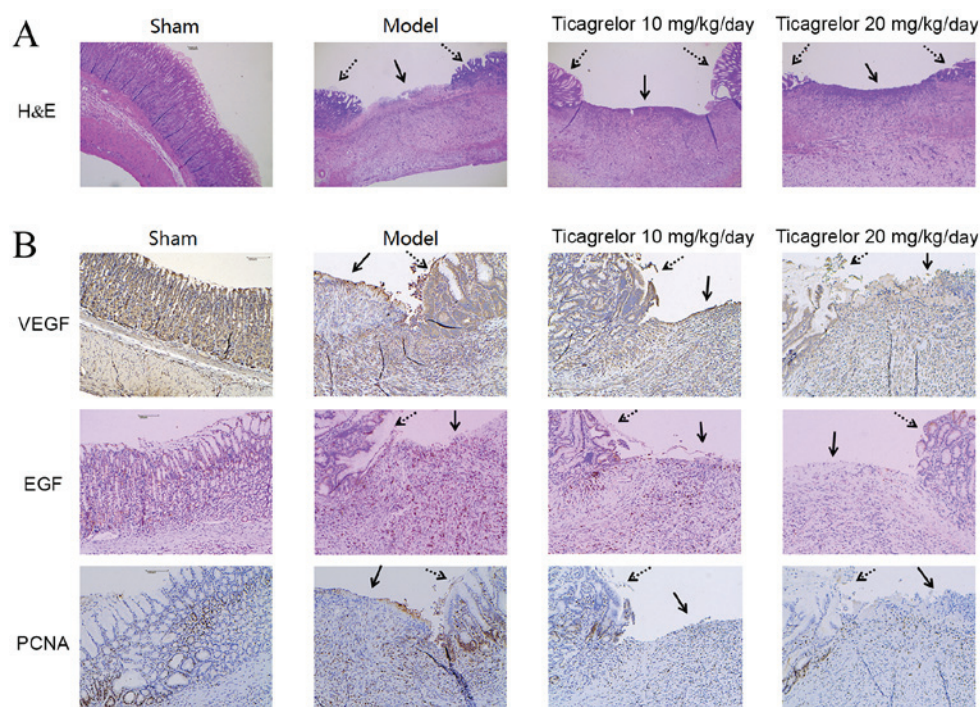


Figure 1. Histological and immunohistochemical analysis of gastric mucosal tissues of rats in the various groups. The filled arrow indicated the ulcer area internal. The dashed arrow indicated the ulcer edge. (A) Hematoxylin and eosin staining (magnification, x40), and (B) immunoreactivity for VEGF, EGF and PCNA antibodies (magnification, x100) are shown. VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; PCNA, proliferation cell nuclear antigen.

Statistical analysis. Results are expressed as the mean \pm standard deviation. SPSS version 11.5 (IBM Corp., Armonk, NY, USA) was used to conduct analysis. The data were analyzed by one-way analysis of variance followed by Tukey's multiple comparison. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Ticagrelor effect on gastric ulcer healing. Delayed rat gastric ulcer healing on day 8 after ulcer induction was observed in the ticagrelor-treated groups when compared with the model group. Oral administration of ticagrelor (10 or 20 mg/kg/day) significantly increased the area of chronic ulcer (26 ± 6 and 27 ± 6 mm², respectively) compared with the model group (20 ± 5 mm², $P < 0.05$). However, there was no significant difference between the low and high dose ticagrelor groups ($P > 0.05$).

Histological analysis. Histological analysis by H&E staining was performed to investigate the absence (ulcer area internal; filled arrow in Fig. 1A) or presence of the epithelial layer (ulcer edge; dashed arrow in Fig. 1A). In the model group, superficial mucosal necrosis was observed, and the structures of the glands, muscularis mucosa, submucosa and muscular were damaged. In addition, moderate amount of inflammatory cells infiltrated the basal layer of the ulcers, and a thin layer of granulation tissues with angiogenesis at the ulcer base was found. In the low and high dose ticagrelor groups, the size of the ulcer and the length of ruptured muscularis mucosa were larger when compared with the model group. Increased mucosal necrosis and inflammatory cell infiltration were also observed in the ticagrelor groups compared with model group.

Immunohistochemical analysis for VEGF, EGF and PCNA.

As shown in Fig. 1B via semi-quantitative analysis using Image-Pro Plus 6.0, VEGF, EGF and PCNA were rarely expressed in sham group. However, cells in the ulcer edges and ulcer beds were strongly stained by the anti-VEGF antibody in the model group, while ticagrelor treatment decreased the expression of VEGF. Upon staining with anti-EGF antibody, only a small number of EGF immunopositive cells were observed in the bottom of the oxyntic glands in the gastric tissues of the sham group. Ulceration led to increased expression of EGF in the damaged epithelial portion, as well in the foveolar and glandular parts in the model group. Ticagrelor treatment decreased the number of EGF-positive cells, revealing decreased EGF expression, as compared with the sham group (Fig. 1A).

A moderate number of PCNA-positive nuclei in the layer adjacent to the ulcer were identified in the model group. The ticagrelor-treated groups demonstrated a lower percentage of labeled nuclei, suggesting that ticagrelor treatment delayed cell proliferation in the area of gastric mucosal healing.

Effect of ticagrelor on VEGF and EGF level at the ulcer margin. There were significant increases in the mucosal VEGF and EGF levels at the ulcer margin when compared with those of the sham mucosa, as determined by ELISA. By contrast, ticagrelor treatment dose-independently decreased mucosal VEGF and EGF levels at the ulcer margin when compared with those of the model group (Fig. 2).

Effect of ticagrelor on protein expression of ERK, P38 and P65 at the gastric ulcer margin. Ulcer induction markedly increased the protein expression levels of P-ERK, P-P38

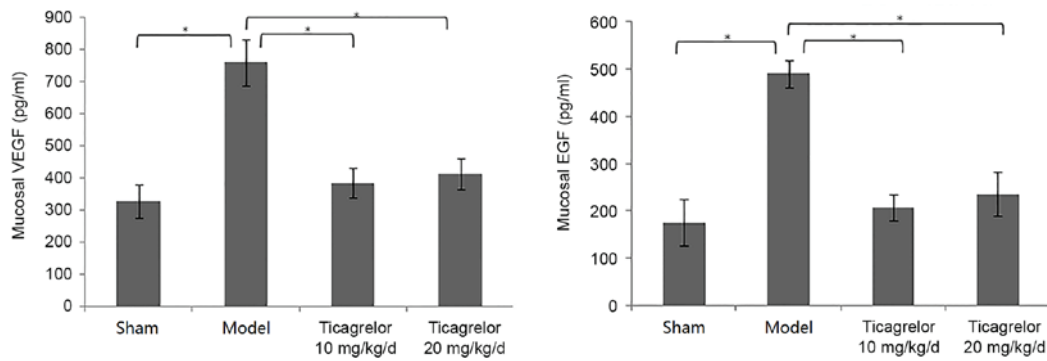


Figure 2. Effects of ticagrelor treatment (10 or 20 mg/kg/day, intragastrically) on the mucosal VEGF and EGF levels at the ulcer margin on day 8 after ulcer induction, as determined by ELISA. Values are presented as the mean \pm standard deviation (n=8 rats/group). *P<0.05. VEGF, vascular endothelial growth factor; EGF, epidermal growth factor.

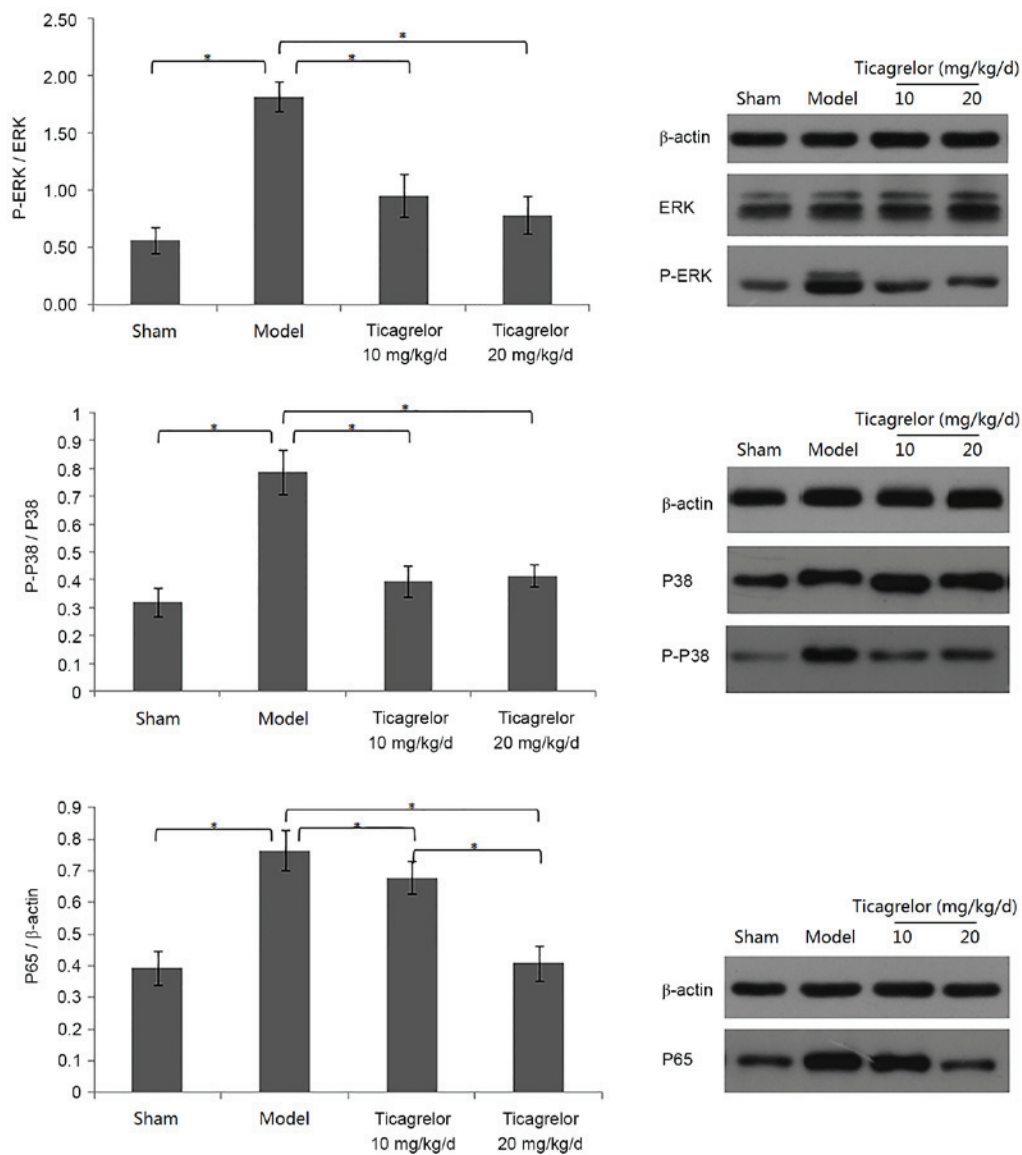


Figure 3. Effects of ticagrelor on the protein expression levels of ERK, P-ERK, P38, P-P38 and nuclear factor- κ B P65 at the ulcer margin on day 8 after ulcer induction, as determined by western blot analysis. Values are presented as the mean \pm standard deviation (n=8 rats/group). *P<0.05. ERK, extracellular signal-regulated kinase; P-, phosphorylated.

and P65 at the ulcer margins when compared with those in sham mucosa (P<0.05; Fig. 3). However, ticagrelor treatment

significantly inhibited the ulcer-induced expression levels of P-ERK, P-P38 and P65 at the ulcer margin compared with the

model group ($P < 0.05$; Fig. 3). And the expression of P65 in high-dose group was significantly lower than that in low-dose group ($P < 0.05$).

Discussion

Accumulated evidence supports the benefits of antiplatelet therapies as a primary and secondary treatment for cardiovascular disease. However, the use of antiplatelet agents has certain risks, in particular, gastrointestinal complications, such as ulceration and resultant bleeding. Previous studies have demonstrated that clopidogrel, a platelet ADP P2Y₁₂ receptor antagonist, evidently increases gastrointestinal bleeding (6) and delays acetic acid-induced gastric ulcer healing by attenuating gastric epithelial cell proliferation via inhibiting the expression of EGF and the phosphorylation activation of the ERK pathway (11). Ticlopidine, another ADP P2Y₁₂ receptor antagonist, delays acetic acid-induced gastric ulcer healing and impairs the angiogenesis and healing of rat gastric ulcer by suppressing the release of VEGF (12). However, no previous studies have reported the possible suppression effects of ticagrelor, a relatively novel P2Y₁₂ receptor antagonist, on gastric ulcer healing.

The results of the present study indicated that ticagrelor delayed gastric ulcer healing in a dose-independent manner through reduction of gastric epithelial cell proliferation and angiogenesis. The mean ulcer sizes were larger in the ticagrelor-treated groups when compared with the model group. PCNA is a nuclear protein associated with the cell cycle, whose appearance is correlated with the proliferation state of the cell (11). In the current study, ticagrelor treatment decreased PCNA expression adjacent to the ulcer, which indicated that ticagrelor inhibited cell proliferation. These findings were in line with previous clinical suggestions that ticagrelor is not sufficiently safe for the gastrointestinal mucosa of patients at high risk of developing peptic ulcers (8).

Ulcer healing is a complex process of tissue regeneration, which involves cell proliferation and angiogenesis, ultimately leading to scar formation (13). All these processes are controlled by various growth factors (14). Ulceration triggers the expression of genes encoding for the growth factors, such as EGF and VEGF, in the cells lining the mucosa of the ulcer margin (14). These growth factors produced locally activate epithelial cell migration and proliferation via autocrine and/or paracrine actions. EGF, a polypeptide growth factor, exerts a wide variety of biological effects including the promotion of proliferation, and is essential for gastric ulcer repair and healing (14). Within 3 days after ulcer formation, cells lining the gastric glands in the ulcer margin undergo dedifferentiation, express EGF and its receptor, and actively proliferate (14). EGF in turn locally stimulates cell proliferation, migration and hence ulcer healing (14). Exogenous EGF has been demonstrated to significantly accelerate experimental gastric ulcer healing (15). Angiogenesis is essential for the healing of chronic gastric ulcers, and VEGF is a fundamental regulator of angiogenesis. Following ulceration, cells lining the mucosa of the ulcer margin express VEGF, which then binds to its specific receptors and triggers endothelial cell proliferation, migration and microvascular tube formation and angiogenesis (14). Exogenous VEGF has been observed to markedly

accelerate healing of experimental gastric ulcers in rats (16). In the present study, ticagrelor administration reduced EGF and VEGF expression levels, and thus inhibited gastric epithelial cell proliferation and angiogenesis.

MAPKs, including the subgroups of ERK1/2, ERK5, p38 and c-Jun N-terminal kinase, are important signal transducer for cell survival and can regulate several cellular processes including proliferation, differentiation, survival and apoptosis (17). Among them, ERK1/2 mediated EGF-induced cell proliferation and VEGF-induced angiogenesis, while p38 functions as a key component in the induction of cell growth processes, cell differentiation, apoptosis and cellular responses to inflammation. A previous study on experimental gastric ulcers in rats has demonstrated that ulceration triggers increased expression of EGF in epithelial cells of the ulcer margin, and that healing and regeneration of the epithelial component of gastric ulcers involves the activation of the MAPK (ERK1/2) signal transduction pathway (14). Blockage of this pathway with a specific inhibitor of EGF-R kinase clearly delayed gastric ulcer healing (14). The present study revealed that ulcer induction activated the EGF-MAPK (ERK1/2 and P38) signal transduction pathways, while ticagrelor inhibited ulcer-induced the activation of these pathways. Meanwhile, VEGF also has the function of activating the MAPK (ERK1/2) kinase signal transduction pathway that triggers angiogenesis. Previous studies have demonstrated that activation of the MAPK (ERK1/2) kinase signal transduction pathway is crucial for VEGF-induced stimulation of angiogenesis, and that nonsteroidal anti-inflammatory drugs may interfere with angiogenesis by inhibiting MAPK (ERK1/2) pathway (18). The present study revealed that ticagrelor impaired the angiogenesis and healing of rat gastric ulcer by suppressing the release of VEGF via the inhibition of the MAPK (ERK1/2 and P38) signal transduction pathway.

NF- κ B signaling regulates vascular homeostasis *in vivo*. Treatment of zebrafish embryos with NF- κ B inhibitors provokes vascular leakage and alters vessel morphology (19). Following ulcer induction, thrombi in the submucosal vessels lead to ischemic mucosal necrosis. In the process of ulcer healing, inflammatory cell aggregation, oxidative stress and cell proliferation exacerbate hypoxia in the ulcer. Hypoxia is one of the best characterized stimuli for the induction of VEGF production by a variety of cells and tissues, while VEGF expression is upregulated by hypoxia-induced mitogenic factor through activation of the NF- κ B pathway (20). In the present study, ulcer induction markedly induced phosphorylation of NF- κ B P65, whereas administration of ticagrelor impaired phosphorylation of these molecules.

The low-dose of ticagrelor used in the present study (10 mg/kg/day in rats) is similar to the dose used in previous studies (10) and in clinical practice (90 mg twice a day for humans) (7). Furthermore, 20 mg/kg/day was applied as the high-dose in the present study. Therefore, the inhibitory effect of ticagrelor on gastric ulcer healing in the current study may resemble a real-life situation.

In conclusion, the present study revealed that ticagrelor administration delayed gastric ulcer healing in rats in a dose-independent manner. The possible underlying mechanisms were associated with the functions of ticagrelor in attenuating the expression levels of VEGF and EGF, as well as

suppressing the phosphorylation activation of ERK1/2, p38 and NF- κ B P65. Finally, the gastric epithelial cell proliferation and angiogenesis were inhibited following ticagrelor treatment.

Acknowledgements

This study was supported by the Natural Science Fund of Health and Family Planning Commission of Wuhan Municipality, China (grant nos. WX17D12 and WX12C32).

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