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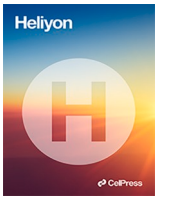

A2BR facilitates the pathogenesis of H. pylori-associated GU by inducing oxidative stress through p38 MAPK phosphorylation

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ABSTRACT

Gastric ulcers significantly impact the quality of life of patients, the pathogenesis of which is closely associated with *Helicobacter pylori* (HP) infection. Oxidative stress is involved in the pathological mechanism of gastric ulcers. Recently, adenosine A2B Receptor (A2BR) was reported to activate the p38MAPK pathway. However, the role of A2BR in gastric ulcers remains unknown. In the present study, the biological function of A2BR in HP-induced gastric ulcers was investigated to explore novel targets for gastric ulcers. GES-1 cells were infected with HP, followed by incubation with 10 μM BAY60-6583 (A2BR agonist) and 25 nM PSB1115 (A2BR antagonist). In HP-infected GES-1 cells, an increased apoptotic rate, enhanced migration ability, excessive release of reactive oxygen species (ROS), increased malondialdehyde (MDA) levels, and decreased superoxide dismutase (SOD) activity were observed, accompanied by the activation of p38MAPK signaling, which were dramatically aggravated by BAY60-6583 and alleviated by PSB1115. In animal experiments, rats were treated with 2 mg/kg BAY60-6583 and 10 mg/kg PSB1115, followed by gastric ulcer modeling 30 min later. In HP-infected rats, increased ulcer area, elevated pepsin activity, increased hematoxylin and eosin (HE) pathological scores, increased MDA levels, and decreased SOD activity were observed, which were further aggravated by BAY60-6583 and ameliorated by PSB1115. Finally, the effects of A2BR activation on apoptosis, migration, oxidative stress, and p38MAPK signaling in HP-infected GES-1 cells were reversed by an inhibitor of the p38MAPK pathway. Collectively, A2BR facilitated the pathogenesis of HP-induced gastric ulcers by inducing oxidative stress through p38MAPK activation.

1. Introduction

Peptic ulcer is a common gastrointestinal disease with a relatively high morbidity of approximately 10 %–20 % [1]. According to the location of the ulcer site, peptic ulcers can be divided into gastric ulcers and duodenal bulb ulcers. Recent studies have confirmed that *Helicobacter pylori* (HP) is one of the main inducers of the onset and recurrence of peptic ulcers, while HP infection is reported in 70 %–80 % of gastric ulcer patients [2]. HP infection induces a variety of different gastrointestinal diseases, including gastric ulcers, and eradication of HP is an important method to treat gastric ulcers [3]. Once Hp enters the stomach, the gastric acid is neutralized due to its urease activity. Under the assistance of flagella, Hp moves on the surface of gastric epithelial cells and interacts with the cell receptors of the host through bacterial adhesins, leading to host damage as HP releases certain effector proteins or toxins, which finally

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triggers the development of gastric ulcers [4]. A series of complications are triggered by gastric ulcers, including upper gastrointestinal bleeding, perforation, pyloric obstruction, and cancerization, which pose significant threats to human health [5]. Therefore, it is crucial to explore the pathogenesis of GU and explore efficient strategies for the treatment of GU.

Oxidative stress is a common pathological process involved in the development of multiple systemic diseases, such as rheumatic diseases, cardiovascular diseases, neuronal degeneration, diabetes, and malignant tumors [6]. The mechanism between oxidative stress and gastric epithelial cell proliferation, necrosis, and apoptosis is complex. Studies have confirmed that oxidative stress can be induced by various gastric mucosal stimulations, leading to DNA damage, which is the key factor contributing to atrophy and intestinal metaplasia [7]. For instance, studies have found that oxidative stress is an important pathological mechanism of gastric ulcers induced by HP [8]. Therefore, oxidative stress might be a promising target for the treatment of gastric ulcers.

Adenosine (Ado) is a regulatory metabolite of purine nucleoside that shows a wide range of biological functions by binding with different adenosine receptors (ARs) and transmitting biological signals [9]. ARs are reported to be coupled to G proteins [10] with four subtypes, A1, A2A, A2B, and A3, among which A2BR shows the lowest affinity for adenosine. Arin RM et al. confirmed through animal experiments that a functional A2BR protein is located in rabbit gastric mucosal parietal cells, which stimulates gastric acid secretion after coupling with Gs protein [11]. It has been reported that the homology of the amino acid sequence of the human A2BR protein and rabbit A2BR protein is 93 %, showing a high similarity [12]. The P38 MAPK signaling pathway is reported to be involved in the activation of oxidative stress and plays an important induction role in the formation of oxidative stress [13], which is closely associated with the pathogenesis of gastric ulcers [14]. Recent studies have reported that p38MAPK signaling can be activated by A2BR [15]. Therefore, we speculate that the human A2BR protein may play a similar role in human gastric physiology and that A2BR might be a regulator of the occurrence and development of HP-associated gastric ulcers in children. In the present study, we explored the biological function of A2BR in the process of HP-induced gastric ulcers and the potential regulatory mechanism.

2. Materials and methods

Antibodies and reagents: GES-1 cells and HP strain 26,695 were obtained from ATCC (Virginia, USA). BAY60-6583 and SB203580 were purchased from MCE, while PSB1115 was purchased from GlpBio. The antibody against p38 was obtained from Affinity, while the antibody against p-p38 and the secondary antibody used in the Western blotting assay were purchased from CST.

Establishment of HP-infected human gastric mucosa cells: GES-1 cells were seeded in 6-well plates at a density of 3×10^5 cells/well in FBS-free DMEM. HPs were introduced into DMEM at a light density of 0.6 under 600 nm, followed by addition to the culture medium containing GES-1 cells at a cell number ratio of 100:1 (HP: GES-1 cells). After incubation at 37 °C and 5 % CO_2 for 24 h, an inverted microscope (AE2000, Motic, China) was utilized for the observation of HP-infected GES-1 cells.

Grouping of in vitro assays: Four groups were established in the in vitro assay: Control, HP, HP + BAY60-6583, and HP + PSB1115. In the control group, GES-1 cells were cultured under normal conditions. In the HP group, GES-1 cells were infected with HP for 24 h. In the HP + BAY60-6583 group, HP-infected GES-1 cells were incubated with 10 μM BAY60-6583 (MCE, USA), an agonist of A2BR [16], for 24 h. In the HP + PSB1115 group, HP-infected GES-1 cells were incubated with 25 nM PSB1115 (GlpBio, USA), an antagonist of A2BR [17], for 24 h.

Apoptosis analysis: Cells were seeded in 6-well plates and incubated with Annexin V-fluorescein isothiocyanate (556,547; BD Biosciences, USA), followed by the addition of propidium iodide. After 10 min of incubation in the dark, the samples were loaded onto a flow cytometer (Attune NxT, Thermo Fisher, USA) for apoptosis analysis.

Transwell assay: The upper chamber of the Transwell insert (3422; Corning, USA) was implanted with 1.5×10^5 cells cultured in serum-free medium, which were then filled with 20 % FBS-supplemented medium in the lower chamber. After 24 h of incubation, the cells were wiped off of the upper chamber, and those in the lower chamber were stained with crystal violet. Finally, the migrated cells were counted using an optical microscope (AE2000; Motic, China).

Wound healing assay: After GES-1 cells reached confluency, a Pasteur pipette was used to scrape a linear wound, and the scratch width was measured. Following 24 h of incubation, the detached cells and debris were cleared using PBS, and the scratch width was measured to calculate the percentage of wound closure.

DCFH-DA assay for the detection of ROS: The culture medium was absorbed and rinsed with warm PBS twice. Approximately 1 mL of diluted DCFH-DA (1:1000) was added and incubated at 37 °C for 20 min in the dark. Cells were digested with 0.25 % trypsin for 1 min, and the digestion was terminated by adding serum-containing medium. The collected cell precipitate was suspended again, and the fluorescence intensity (excitation wavelength was 500 nm, emission wavelength was 525 nm) was assessed by a fluorescence microplate analyzer (Ts2-FC, Nikon, Japan).

MDA level and SOD activity: The levels of MDA and SOD activity were determined by commercial kits (Nanjing Jiancheng, China) using the TBA method and WST-1 method, respectively, according to the manufacturer's instructions.

Western blot analysis: Cells or tissues were isolated with lysis buffer and further quantified with the BCA method (pc0020, Solarbio, China). SDS–PAGE was used to separate proteins at a 12 % concentration, followed by transferring proteins to a PVDF membrane (10600023, GE Healthcare Life, USA). After blocking, primary antibodies against p38 (1:5000, BF8015, Affinity, USA) and p-p38 (1:2000, 9216S, CST, USA) were incubated at 4 °C for 12 h, followed by introducing the secondary antibody (1:3000, 7072, CST, USA) for 90 min. Finally, the level of proteins was quantified by analyzing bands with ImageJ software.

Establishment of the HP-induced GU model in rats: GU modeling in rats was conducted according to the modified Okabe method [18]. Twenty-four 180–220 g male Sprague Dawley (SD) rats (7–9 weeks old) were obtained from SLAC ANIMAL (Shanghai, China), and eighteen rats were utilized for the establishment of the gastric ulcer model. After fasting for 24 h, rats were anesthetized by an intraperitoneal injection of 30 mg/kg 1 % pentobarbital sodium solution, followed by fixation and shaving of the belly wool. The

abdominal wall was dissected approximately 1.8 cm to the left of the lower ventral midline of the xiphoid process. The stomach was extracted, and the glandular stomach was clamped with mold forceps. Then, 10 μL of 100 % glacial acetic acid was injected into the anterior wall of the gastric antrum under the serous membrane near the muscle layer with a microsyringe, followed by wrapping in the greater omentum. The stomach was then fed into the abdominal cavity, the abdominal wall was sutured layer by layer, and the abdomen was closed. On the third day after the operation, rats were fasted for 12 h and then fed with 2 mL 5 % $NaHCO_3$ by esophagus, followed by injection of 1.5 mL HP strain 26,695 (2×10^8 cfu/mL) by gastric tube 15 min later. The diet was permitted 30 min later. HP injection was conducted once every three days, and was conducted a total of 3 times. The abdominal wall of the rats was dissected, and the stomach was extracted in the sham group, followed by saturation without injection of glacial acetic acid and HP.

Grouping of the in vivo experiment: Rats were divided into 4 groups (n = 6): Sham, Model, Model + BAY60-6583, and Model + PSB1115. Rats in the Model + BAY60-6583 group were intratracheally injected with 2 mg/kg BAY60-6583 half an hour prior to modeling, while rats in the Model + PSB1115 group were intratracheally injected with 10 mg/kg PSB1115 half an hour prior to modeling [19]. Animals in the sham and model groups were intratracheally injected with the same volume of normal saline 30 min

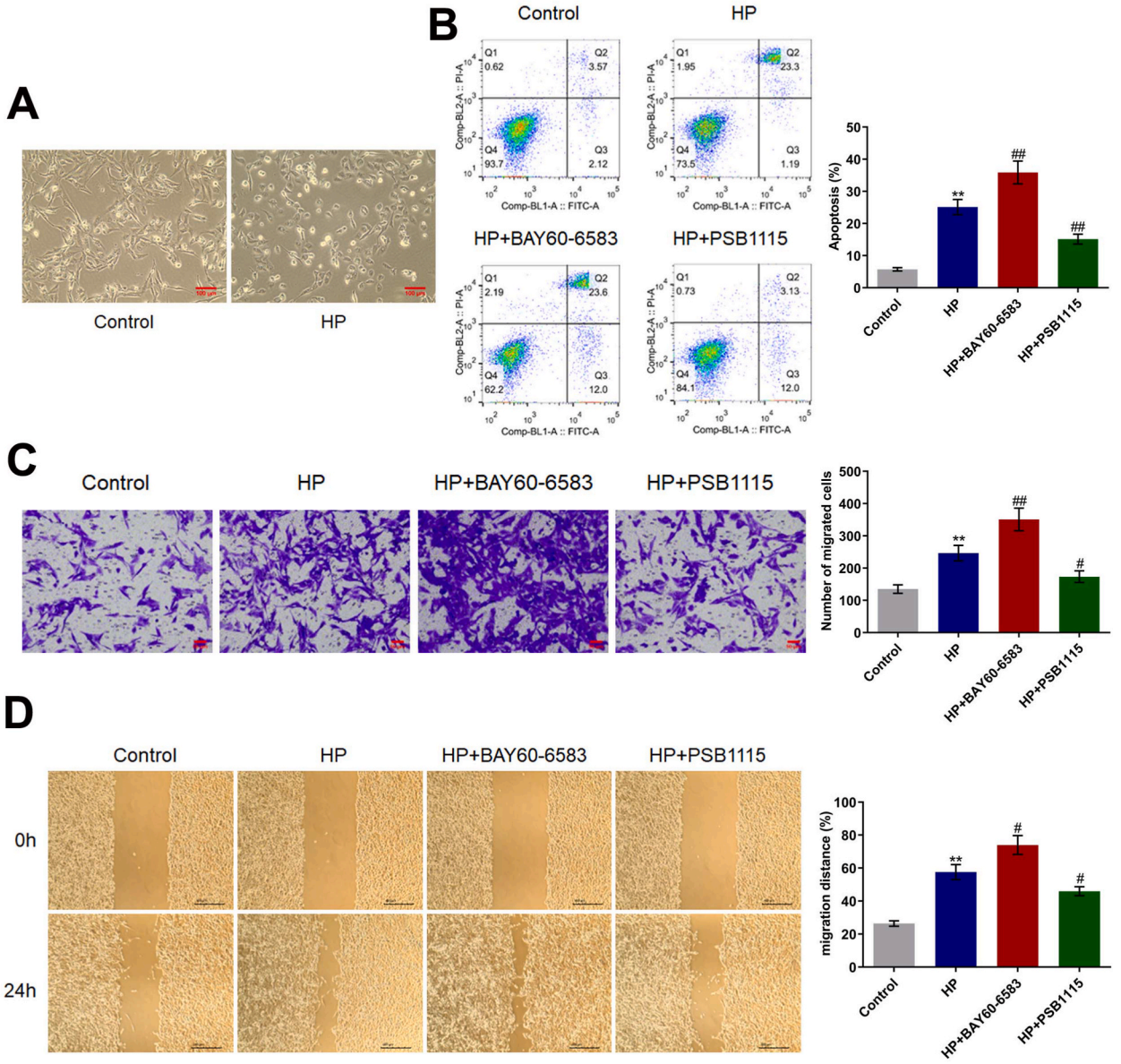

Fig. 1. The apoptosis and migration of HP-infected GES-1 cells were facilitated by A2BR activation. A. The morphology of GES-1 cells was observed under an inverted microscope. GES-1 cells were infected with HP, followed by incubation with 10 μM BAY60-6583 and 25 nM PSB1115. B. Flow cytometry was utilized to measure the apoptotic rate. C. The transwell assay was used to evaluate the migration ability. D. The metastasis of cells was determined using the wound healing assay (**p < 0.01 vs. control, #p < 0.05 vs. HP, ##p < 0.01 vs. HP).

prior to the surgery.

The measurement of ulcer area: The maximum longitudinal diameter and transverse diameter of the ulcer passing through the center of the ulcer were measured using a Vernier caliper, followed by calculating the ulcer area according to the following formula: ulcer area = π × longitudinal diameter/2 × transverse diameter/2.

Pepsin activity measurement: The pepsin activity was determined using the stop-point assay of denatured hemoglobin hydrolysis described previously [20].

HE staining: The obtained gastric mucosa tissues were fixed in 4 % formaldehyde solution for 2 days, followed by gradient dehydration with 70 %, 80 %, 90 %, 95 %, and 100 % alcohol. After clearing twice with xylene, tissues were placed in dissolved paraffin and then kept in a wax solubilizing box for 2 h at 65 °C for embedding. After cooling and solidification, paraffin blocks of tissues were automatically cut in a transverse section in the middle with a microtome at a thickness of 4 μm, while sections were then scalded in hot water, reattached to glass slides and kept in an oven at 58 °C for 4 h. After deparaffinization, sections were stained with hematoxylin for 3 min, differentiated with 1 % hydrochloric acid alcohol for 20 s, and stained with eosin for 5 min. Dehydration was performed with 70 %, 80 %, 90 %, 95 % and 100 % alcohol in turn, and transparency was carried out with xylene and kept for 5 min. Images were obtained using an inverted microplate (NIKON ECLIPSE E100, Nikon, Japan) to observe the pathological changes in gastric mucosa tissues.

Statistical analysis: The data are expressed as the mean ± SD and were analyzed with GraphPad software. One-way ANOVA with Tukey's post hoc test was applied for comparison. $P < 0.05$ was regarded as a significant difference.

3. Results

A2BR activation induced the apoptosis and migration of HP-infected GES-1 cells. First, under an inverted microscope, obvious apoptosis was observed in HP-infected GES-1 cells (Fig. 1A). GES-1 cells were infected with HP, followed by incubation with 10 μM

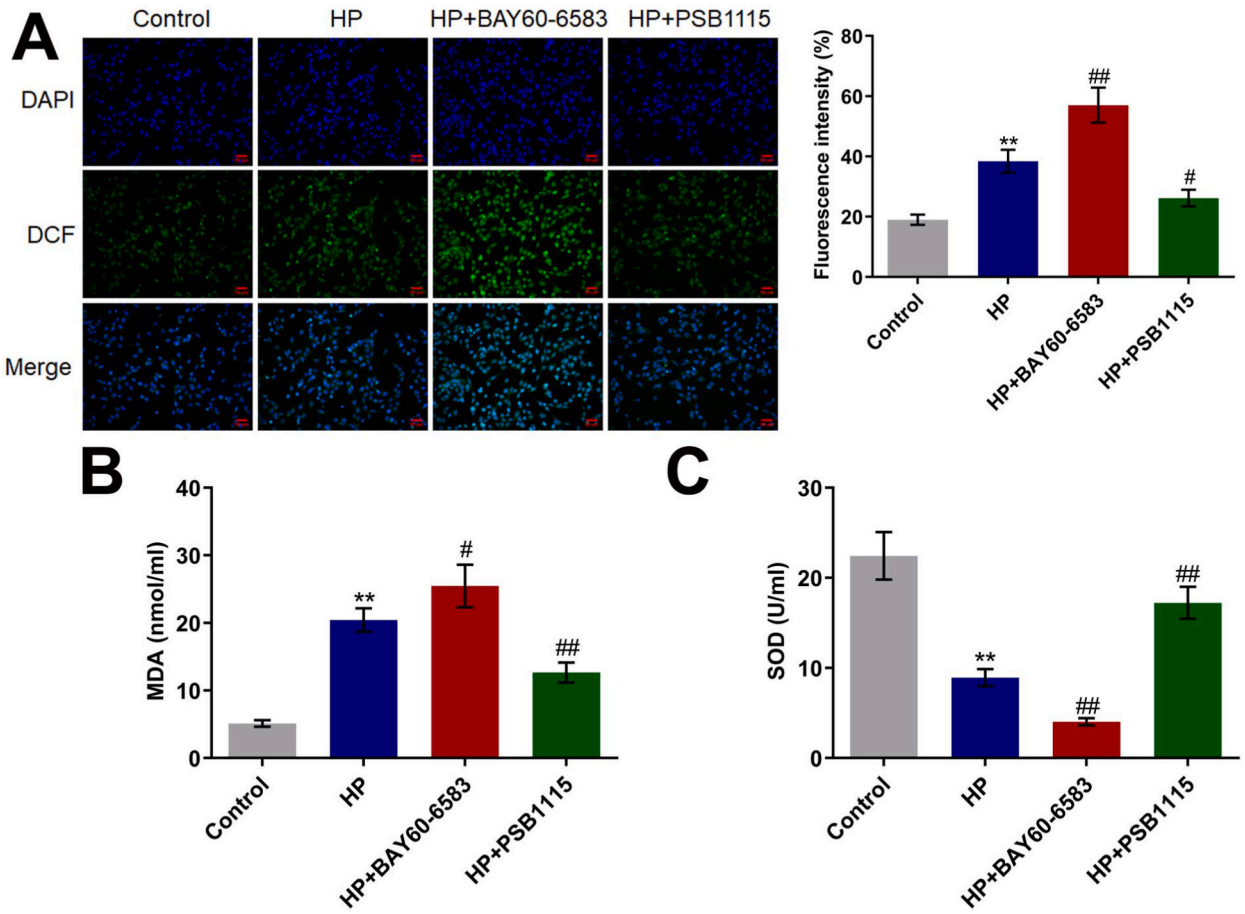

Fig. 2. Oxidative stress in HP-infected GES-1 cells was aggravated by A2BR activation. GES-1 cells were infected with HP, followed by incubation with 10 μM BAY60-6583 and 25 nM PSB1115. A. The ROS level in GES-1 cells was evaluated by the DCFH-DA assay. B. The MDA level was determined by a commercial kit using the TBA method. C. SOD activity was checked by a commercial kit using the WST-1 method (**$p < 0.01$ vs. control, #$p < 0.05$ vs. HP, ##$p < 0.01$ vs. HP).

BAY60-6583 and 25 nM PSB1115. We found that the apoptotic rate (Fig. 1B) was increased from 5.69 % to 24.49 % by HP infection, which was greatly promoted to 35.6 % by BAY60-6583 and declined to 15.13 % by PSB1115. Furthermore, the number of migrated cells (Fig. 1C) in HP-infected GES-1 cells was elevated from 135.0 to 246.7, which was further promoted to 351.0 by BAY60-6583 and repressed to 173.7 by PSB1115. The migration distance of HP-infected GES-1 cells (Fig. 1D) was increased from 26.4 % to 57.6 %, which was further elevated to 74.0 % by BAY60-6583 and reduced to 46.0 % by PSB1115. These data suggested that the apoptosis and migration of HP-infected GES-1 cells were dramatically induced by the activation of A2BR.

A2BR activation aggravated oxidative stress in HP-infected GES-1 cells. We further explored the effect of A2BR on oxidative stress in HP-infected GES-1 cells. The ROS level in GES-1 cells was determined by the fluorescence intensity detected by the DCFH-DA assay (Fig. 2A). The fluorescence intensity dramatically increased from 19.0 % to 38.4 % in HP-infected GES-1 cells, which was further increased to 57.1 % by BAY60-6583 and repressed to 26.2 % by PSB1115. Furthermore, the MDA levels (Fig. 2B) in the control, HP, HP + BAY60-6583, and HP + PSB1115 groups were 5.13, 20.44, 25.48, and 12.67 nmol/mL, respectively. In addition, SOD activity (Fig. 2C) declined from 22.45 U/mL to 8.94 U/mL in HP-infected GES-1 cells, which was reduced to 4.03 U/mL by BAY60-6583 and reversed to 17.24 U/mL by PSB1115. These data revealed that oxidative stress in HP-infected GES-1 cells was aggravated by the activation of A2BR.

A2BR activation activated p38MAPK in HP-infected GES-1 cells: To explore the potential mechanism of A2BR in regulating HP-infected GES-1 cells, the state of the p38MAPK pathway in each group was checked (Fig. 3). No significant difference was observed in the expression level of p38 among the 4 groups. However, p-p38 was dramatically upregulated in HP-infected GES-1 cells, the expression of which was further elevated by BAY60-6583 and inhibited by PSB1115, indicating that the p38MAPK pathway was activated by the activation of A2BR.

A2BR activation aggravated gastric ulcers in HP-treated rats: To further verify the function of A2BR in HP-induced gastric ulcers, rats were treated with 2 mg/kg BAY60-6583 or 10 mg/kg PSB1115, followed by gastric ulcer modeling 30 min later. We found that the ulcer areas (Fig. 4A) in the sham, model, model + BAY60-6583, and model + PSB1115 groups were 0, 25.88, 40.48, and 15.20 mm^2, respectively. Furthermore, pepsin activity (Fig. 4B) was elevated from 9.19 U/mL to 35.18 U/mL in the Model group, which was further increased to 54.15 U/mL by BAY60-6583 and decreased to 18.29 U/mL by PSB1115. The pathological state of gastric mucosa tissues was evaluated by HE staining (Fig. 4C). In the Sham group, the microscopic histological appearance of the gastric tissue and the normal gastric epithelium were observed. Disruption of the superficial region of the gastric gland with loss of epithelial cells, pronounced edema of the submucosa, and degradation of the mucosa were observed in the Model group. Compared to the model group, these pathological changes were further aggravated by BAY60-6583 and alleviated by PSB1115, with relatively higher and lower HE scores, respectively. In addition, the MDA levels in the gastric mucosa tissues (Fig. 4D) in the sham, model, model + BAY60-6583, and model + PSB1115 groups were 1.39, 3.47, 5.61, and 2.28 nmol/mg prot, respectively. Moreover, SOD activity (Fig. 4E) declined from 4.68 U/mg prot to 2.08 U/mg prot in the model group, which was further reduced to 1.09 U/mg prot by BAY60-6583 and reversed to 3.90 U/mg prot by PSB1115. These data revealed that the pathological state of gastric ulcers in HP-treated rats was aggravated by the activation of A2BR, accompanied by the activation of oxidative stress.

A2BR activation activated p38MAPK in HP-treated rats: The state of the p38MAPK pathway in gastric mucosa tissues was further investigated. We found that the level of p-p38/p38 in the Model group was dramatically elevated, and was further increased by BAY60-6583 and repressed by PSB1115 (Fig. 5), indicating a facilitating effect of A2BR activation on the p38MAPK pathway in HP-treated rats.

P38MAPK inhibitor abolished the effect of A2BR on the apoptosis and migration of HP-infected GES-1 cells: To verify that A2BR activation exerted its function in HP-induced gastric ulcers by activating p38MAPK signaling, SB203580, an inhibitor of p38MAPK signaling [21], was cointroduced into HP-infected GES-1 cells with BAY60-6583. HP-infected GES-1 cells were incubated with 10 μM BAY60-6583 in the presence or absence of 5 μM SB203580. We found that the apoptotic rate in HP-infected GES-1 cells was increased from 6.79 % to 19.54 %, which was further elevated to 38.58 % by BAY60-6583. After coincubation with 5 μM SB203580,

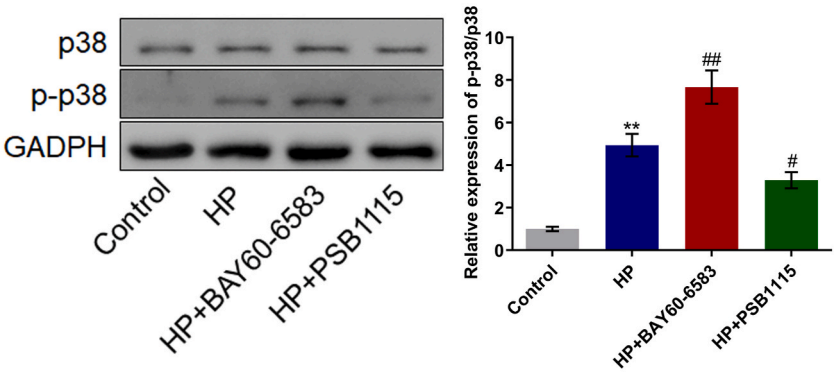

Fig. 3. P38MAPK signaling in HP-infected GES-1 cells was activated by A2BR activation. GES-1 cells were infected with HP, followed by incubation with 10 μM BAY60-6583 and 25 nM PSB1115. The expression levels of p38 and p-p38 were evaluated by Western blotting (**$p < 0.01$ vs. control, #$p < 0.05$ vs. HP, ##$p < 0.01$ vs. HP).

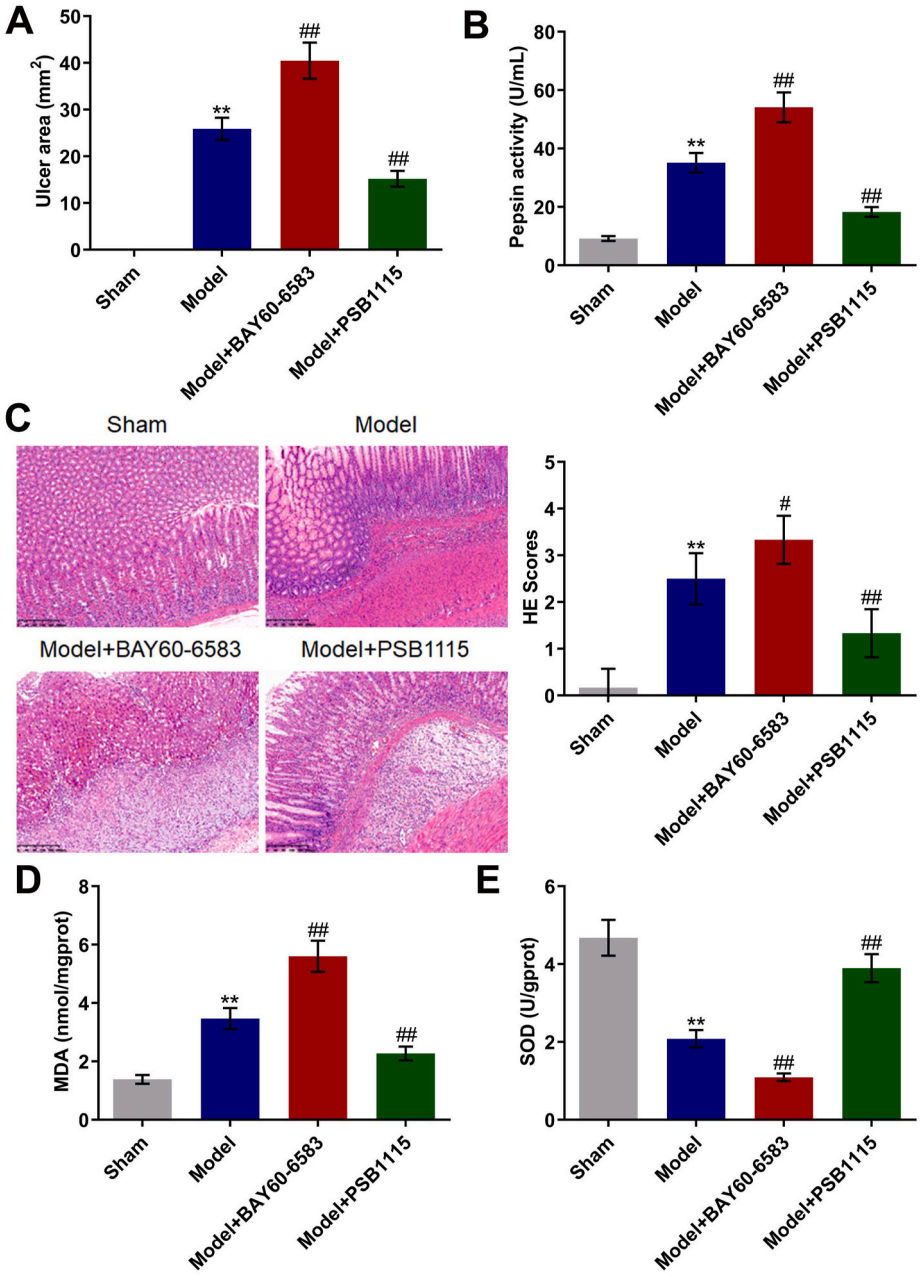

Fig. 4. The pathological state of gastric ulcers in HP-treated rats was aggravated by A2BR activation. Rats were treated with 2 mg/kg BAY60-6583 or 10 mg/kg PSB1115, followed by gastric ulcer modeling 30 min later. A. The ulcer area in each group was calculated. B. The pepsin activity in each group was determined using the stop-point assay of denatured hemoglobin hydrolysis. C. The pathological changes in gastric mucosa tissues were checked by the HE staining assay. D. The MDA level in gastric mucosa tissues was determined by a commercial kit using the TBA method. E. The SOD activity in gastric mucosa tissues was checked by a commercial kit using the WST-1 method (**$p < 0.01$ vs. control, #$p < 0.05$ vs. HP, ##$p < 0.01$ vs. HP).

the apoptotic rate was dramatically reversed to 25.59 % (Fig. 6A). Furthermore, the number of migrated cells in the control, HP, HP + BAY60-6583, and HP + BAY60-6583 + SB203580 groups was 128.3, 220.3, 334.7, and 245.7, respectively (Fig. 6B). In addition, the migration distance in the wound healing assay was greatly elevated from 20/4 %–46.7 % in HP-infected GES-1 cells, which was further increased to 74.8 % by BAY60-6583. After coincubation with 5 μM SB203580, the migration distance was greatly reduced to 54.8 % (Fig. 6C). These results suggested that the effect of A2BR activation on the apoptosis and migration of HP-infected GES-1 cells was greatly abolished by the inhibitor of p38MAPK signaling.

The **p38MAPK inhibitor abolished the effect of A2BR on oxidative stress in HP-infected GES-1 cells:** We further investigated

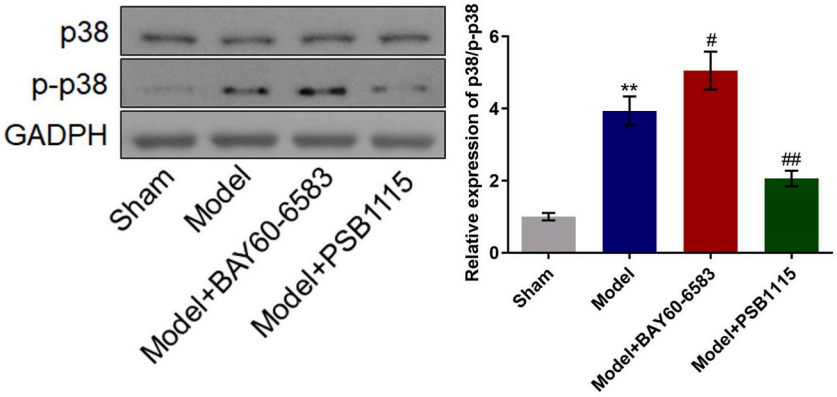

Fig. 5. P38MAPK signaling in HP-treated rats was activated by A2BR activation. Rats were treated with 2 mg/kg BAY60-6583 or 10 mg/kg PSB1115, followed by gastric ulcer modeling 30 min later. The expression levels of p38 and p-p38 in gastric mucosal tissues were evaluated by Western blotting (**$p < 0.01$ vs. control, #$p < 0.05$ vs. HP, ##$p < 0.01$ vs. HP).

the state of oxidative stress in the 4 groups. The fluorescence intensity was increased from 18.6 % to 33.3 % in HP-infected GES-1 cells, which was further elevated to 59.7 % by BAY60-6583. After cotreatment with 5 μM SB203580, the fluorescence intensity decreased to 41.7 % (Fig. 7A). Furthermore, the MDA levels in the control, HP, HP + BAY60-6583, and HP + BAY60-6583 + SB203580 groups were 5.62, 11.28, 25.26, and 18.43 nmol/mL, respectively (Fig. 7B). In addition, SOD activity was reduced from 22.32 U/mL to 17.76 U/mL in HP-infected GES-1 cells, which further decreased to 3.98 U/mL by the administration of BAY60-6583. After cotreatment with 5 μM SB203580, the SOD activity was reversed to 9.48 U/mL (Fig. 7C). These results revealed that the effect of A2BR activation on oxidative stress in HP-infected GES-1 cells was abolished by the inhibitor of p38MAPK signaling.

4. Discussion

In the present study, in vitro and in vivo models of gastric ulcers were established by infecting GES-1 cells and rats with HP, both of which were treated with an agonist and antagonist of A2BR to explore the potential function of A2BR in gastric ulcers. A promising protective effect of the antagonist of A2BR on HP-infected GES-1 cells and rats was observed, while an aggravative function of the A2BR agonist against HP-infected GES-1 cells and rats was observed, suggesting that A2BR might be an important pathological factor inducing HP-triggered gastric ulcers, accompanied by an aggravative effect of A2BR on oxidative stress and p38MAPK signaling. Furthermore, the impact of the A2BR agonist on HP-infected GES-1 cells was abolished by the p38MAPK inhibitor, implying that p38MAPK might be the downstream signaling pathway of A2BR in gastric ulcers.

Recent studies have emphasized the important role of oxidative stress in gastric ulcers [22,23]. Under normal circumstances, oxidation and antioxidant mechanisms in the body are in a steady equilibrium, the imbalance of which contributes to the excessive production of ROS by endogenous and exogenous factors. As a consequence, the steady state of the oxidation/antioxidant defense system is disrupted, and oxidative stress is activated, which involves DNA, lipids, and proteins. Damage to the gastric mucosa is induced by hyperreactive oxygen species in the gastric mucosa [24]. Daives GR et al. [25] found that the ROS level was elevated significantly after infecting gastric antrum mucosa with HP and that the level of ROS was closely associated with the degree of histological changes and HP infection. Experimental results have shown that the MAPK pathway can be activated by excessive released ROS affecting intracellular calcium ion concentrations or activating protein tyrosine kinases, resulting in corresponding physiological reactions. Studies on ulcer models in which TNF-α and IL-6 secretion are inhibited have shown that alcoholic ulcers can be protected by scavenging ROS and preventing apoptosis [26,27]. SOD is a scavenger of superoxide anions, and can transform superoxide anions into oxygen and hydrogen peroxide, reduce the damage of superoxide anions to the human body, reduce the effect of free radicals on unsaturated fatty acids, and repress the generation of lipid peroxides. The amount and activity of SOD in the gastric mucosa is beneficial for alleviating the potential damage to the gastric mucosa caused by bacteria [28]. Therefore, SOD is an important protective factor in the gastric mucosa. Endogenous antioxidant molecules, including SOD, GSH and CAT, play an important role in resisting ROS-activated oxidative stress injury [29,30]. MDA reflects the degree of lipid peroxidation in the body, which can be considered a reliable indicator of oxidative stress that reflects the severity of cell damage by oxygen free radicals [31,32]. Herein, significant oxidative stress was triggered in HP-infected GES-1 cells, which was in accordance with results reported by Xie [33]. Furthermore, in the HP-induced gastric ulcer rat model, the ulcer pathological changes were accompanied by activated oxidative stress in the gastric mucosa, which was consistent with the report by Ramakrishna [34]. We found that the pathological changes in both HP-infected GES-1 cells and gastric ulcer rats were dramatically aggravated by the activation of A2BR, accompanied by the exacerbation of oxidative stress, while the opposite results were observed after the administration of an A2BR inhibitor. These results suggest that A2BR is potentially involved in the pathogenesis of gastric ulcers, which might be closely associated with oxidative stress.

MAPK is a serine/threonine protein kinase, and the MAPK pathway is an acellular signal transduction pathway that plays an important role in the control of inflammatory processes. MAPK signaling can be activated by LPS, which includes three classic

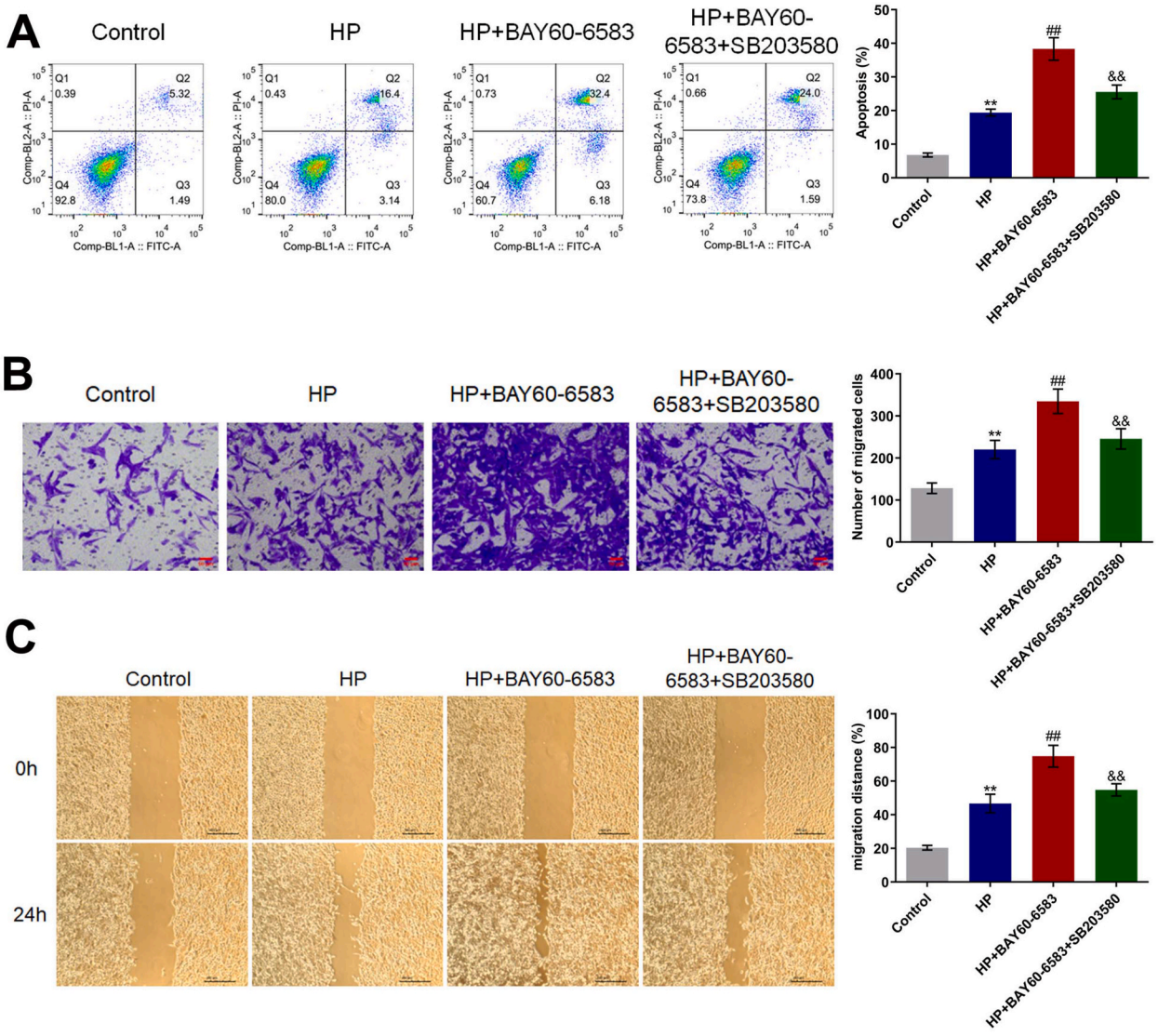

Fig. 6. The effect of A2BR activation on the apoptosis and migration of HP-infected GES-1 cells was abolished by SB203580. HP-infected GES-1 cells were incubated with 10 μM BAY60-6583 in the presence or absence of 5 μM SB203580. A. Flow cytometry was utilized to measure the apoptotic rate. B. The transwell assay was used to evaluate the migration ability. C. The metastasis of cells was determined using the wound healing assay (**p < 0.01 vs. control, ##p < 0.01 vs. HP, &&p < 0.01 vs. HP + BAY60-6583).

pathways: the extracellular signal regulated kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK) pathway, and the p38MAPK pathway. All three pathways are reported to affect a series of biological cellular reactions, such as proliferation, differentiation, transformation, and apoptosis, mostly by affecting gene transcription and regulation. It has been confirmed that tight junction permeability can result from increased p38MAPK activity and that damage to the tight junction barrier in different types of epithelial cells induced by stimuli can be protected by p38 inhibitors [35]. It has been reported that gastric mucosa inflammation may be related to the activation of the MAPK pathway [36]. Slomiany [37] found that in an HP-induced gastritis model, excessive release of cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) was induced by the activation of the MAPK/ERK pathway. Both cyclooxygenase-2 and iNOS are involved in the processing of gastric mucosal inflammatory lesions. Activation of MAPK cascades can be found in HP-infected gastric epithelial cells, which may act as an upstream signal to activate NF-κB and activator protein-1 (AP-1) in HP-infected gastritis [38]. Furthermore, by regulating p38MAPK activity, an inhibitory effect against gastric ulcers was observed. Zheng reported that jia wei wu qi san alleviated gastric ulcers by regulating p38MAPK signaling [39]. Sallam claimed that the alleviation of gastric ulcers by olmesartan was associated with p38MAPK signaling [14]. In the present study, p38MAPK signaling was dramatically activated in HP-infected GES-1 cells, which confirmed the observation reported by Liu [40]. Furthermore, activated p38MAPK signaling was also observed in an HP-induced gastric ulcer rat model. After the introduction of an A2BR agonist, p38MAPK signaling in both HP-infected GES-1 cells and HP-induced gastric ulcer rats was further activated. In addition, the effects of A2BR

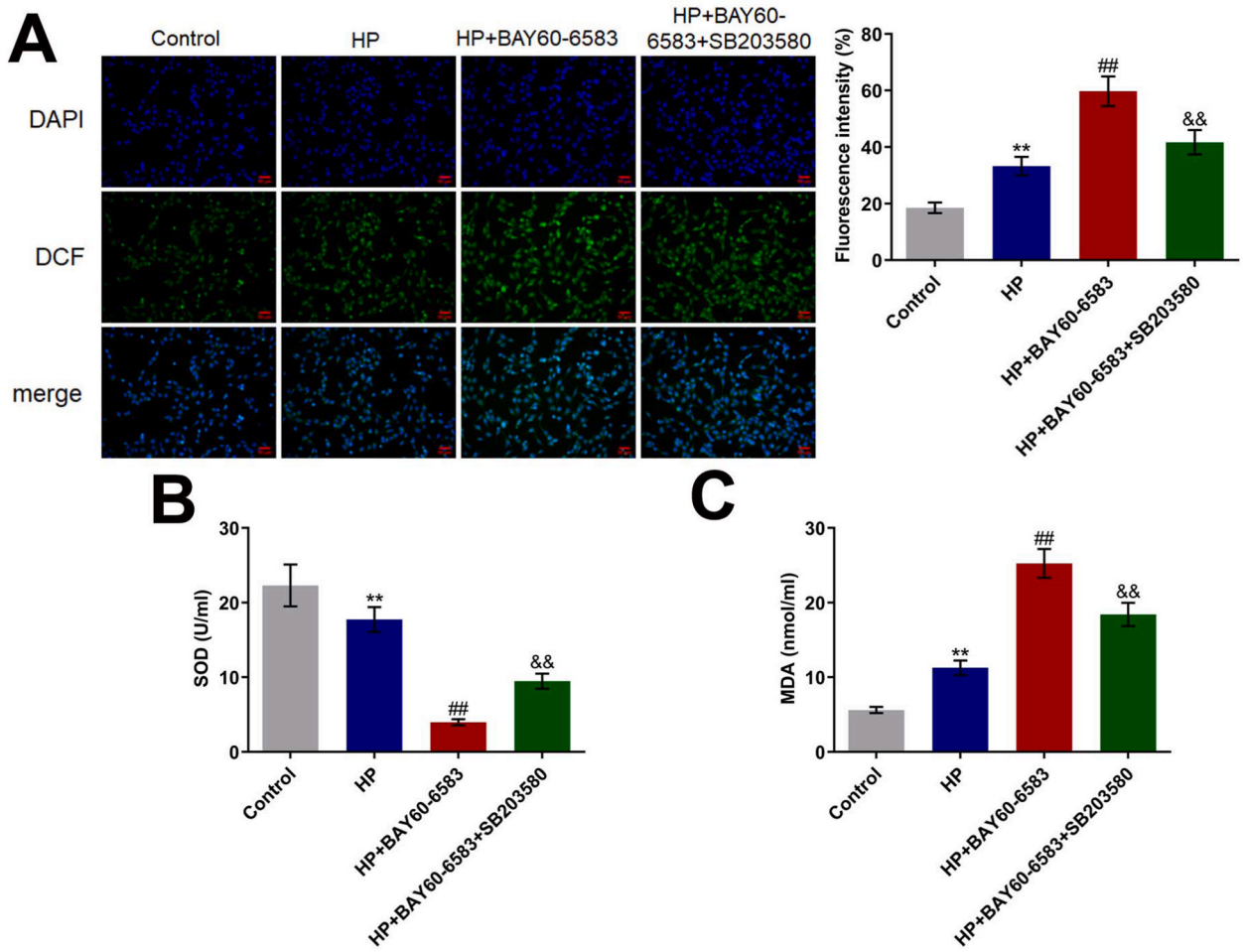

Fig. 7. The effects of A2BR activation on oxidative stress in HP-infected GES-1 cells were abolished by SB203580. HP-infected GES-1 cells were incubated with 10 μM BAY60-6583 in the presence or absence of 5 μM SB203580. A. The ROS level in GES-1 cells was evaluated by the DCFH-DA assay. B. The MDA level was determined by a commercial kit using the TBA method. C. SOD activity was checked by a commercial kit using the WST-1 method (**$p < 0.01$ vs. control, ##$p < 0.01$ vs. HP, &&$p < 0.01$ vs. HP + BAY60-6583).

activation on apoptosis, migration, oxidative stress, and p38MAPK signaling in HP-infected GES-1 cells were reversed by an inhibitor of the p38MAPK pathway, suggesting that A2BR is involved in the pathogenesis of gastric ulcers, possibly by inducing oxidative stress through activating p38MAPK signaling. In our future work, the protective effect of PSB1115 on gastric ulcers will be further verified using different animal models, and the safety evaluation of PSB1115 will be conducted in rats and dogs to verify the feasibility of treating clinical gastric ulcer patients using PSB1115. Furthermore, the stimulation of gastric injury by HCl might be associated with the activation of p38MAPK [41], and we suspect that the inducing function of p38MAPK on gastric ulcers might be associated with the secretion of HCl, which will be addressed in future work.

Collectively, our data reveal that A2BR might facilitate the pathogenesis of HP-induced gastric ulcers by inducing oxidative stress through p38MAPK activation.

Ethic statements

All animal experiments involved in this manuscript were authorized by the animal experimentation ethical committee of Zhejiang Yingyang Pharmaceutical Research and Development Co., LTD (Certificate No. ZJEY-20220124-03).

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Weihong Tang: Data curation, Investigation, Project administration, Writing – original draft. **Minchang Guan:** Data curation, Formal analysis, Methodology. **Ze Li:** Formal analysis, Investigation, Software, Visualization. **Wei Pan:** Data curation, Investigation, Software, Visualization. **Zhongmin Wang:** Conceptualization, Funding acquisition, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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