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Involvement of glutamate–cystine/glutamate transporter system in aspirin-induced acute gastric mucosa injury

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ABSTRACT

Large-dose or long-term use of aspirin tends to cause gastric mucosa injury, which is recognized as the major side effect of aspirin. It has been demonstrated that glutamate exerts a protective effect on stomach, and the level of glutamate is critically controlled by cystine/glutamate transporter (Xc^-). In the present study, we investigated the role of glutamate–cystine/glutamate transporter system in aspirin-induced acute gastric mucosa injury in vitro and in vivo. Results showed that in human gastric epithelial cells, aspirin incubation increased the activity of LDH and the number of apoptotic cells, meanwhile down-regulated the mRNA expression of Xc^- accompanied with decreased glutamate release. Similar results were seen in a rat model. In addition, exogenous L-glutamate attenuated the gastric mucosa injury and cell damage induced by aspirin both in vitro and in vivo. Taken together, our results demonstrated that acute gastric mucosa injury induced by aspirin is related to reduction of glutamate–cystine/glutamate transporter system activity.

1. Introduction

Aspirin is one of the most widely used drugs, a typical antipyretic–analgesic and anti-inflammatory medication [1,2]. However, clinical practices have demonstrated that large-dose or long-term use of aspirin tends to induce gastric mucosa injury, gastric hemorrhage, and even gastric perforation [3]. Previous studies indicate that acute gastric mucosa injury induced by aspirin is ascribed to inhibition of prostaglandin production [4]. Recently, it was reported that exogenous glutamate possessed a protective effect on cold stress-induced gastric ulcer [5,6]. Furthermore, based on the fact that aspirin inhibits production of prostaglandin which can regulate glutamate transportation [7,8], we hypothesize that aspirin-induced acute gastric mucosa injury may involve the glutamate pathway.

Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (CNS), it is closely associated with spatial learning and hippocampus-dependent memory processes [9]. Moreover, the role of glutamate in peripheral organs including lung, bone and stomach are gradually revealed [10]. A proper level of glutamate is crucial for keeping its function and the extracellular level of glutamate is critically controlled by cystine/glutamate transporter (Xc^- system) which consists of heavy chain 4F2hc and light chain xCT. Xc^- system can transport one molecule cystine into the cell and release one molecule glutamate outside at the same time [11–13].

It has been documented that aspirin inhibits the release of glutamate via reducing glutamate transporter activity and plays a protective role in neurons [14]. Recently, studies have reported that glutamate and its receptors are also involved in acute gastric mucosa injury induced by some factors such as cold stress [5,6,15]. It is probable that glutamate-cystine/glutamate transporter system is involved in aspirin-induced gastric mucosa injury.

The purpose of our present study was 3-fold. Firstly, we determined the changes of cystine/glutamate transporter activity in aspirin-induced acute gastric mucosa injury. Secondly, we explored the special role of two subunits of cystine/glutamate transporter mediating aspirin-induced injury. Thirdly, we investigated the protective effect of glutamate in aspirin-induced gastric mucosa injury.

2. Materials and methods

2.1. Animals

Experiments were performed with male Sprague–Dawley rat weighing 250–300 g, which were allowed free access to water and food. Our study was carried out in accordance with the

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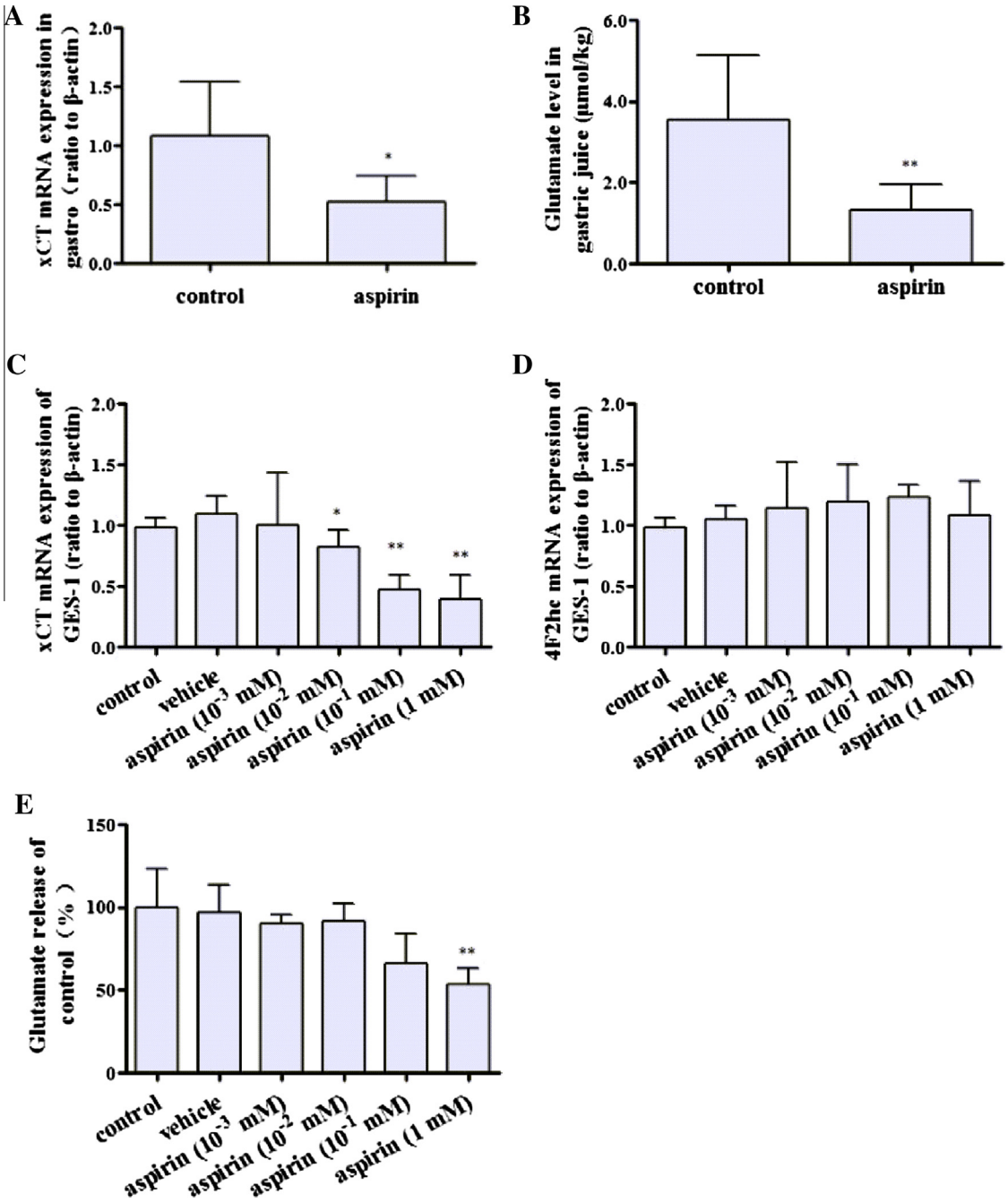

Fig. 1. Aspirin decreases glutamate level and mRNA expression of cystine/glutamate transporter. (A) Cystine/glutamate transporter light chain xCT mRNA expression in gastro ($n = 8$); (B) glutamate level of gastric juice in rats ($n = 8$); (C) xCT mRNA expression of GES-1 ($n = 3$); (D) Cystine/glutamate transporter heavy chain 4F2hc mRNA expression of GES-1 ($n = 3$); (E) glutamate level released from GES-1 (ratio to control, $n = 3$). All data are expressed as mean ± SD. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control. L-glu is L-glutamate, GES-1 is human gastric epithelial cells.

Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

2.2. Induction of gastric lesions

Gastric lesions was induced by intragastric administration of aspirin (400 mg/kg) suspended in CMC-Na (0.5%). All animals were divided into four groups: control-group rats were received 0.5% CMC-Na equivalent to the volume of aspirin only; aspirin-group rats were given aspirin only; others were pretreated with L-glutamate (3 mg/kg or 6 mg/kg). Six hours later, animals were sacrificed and the stomachs were excised. Macroscopically visible gastric damage was measured according to GUTH, and the area of the lesions was summed up.

1.3. Collecting gastric secretion

The pylorus was ligated under chloral hydrate (10%) anesthesia and the stomach was excised 6 h after the ligation. The gastric contents were collected and centrifuged at 1000 r/min for 10 min. The volume of gastric juice (supernatant) was measured. We examined the release of glutamate in control and aspirin groups.

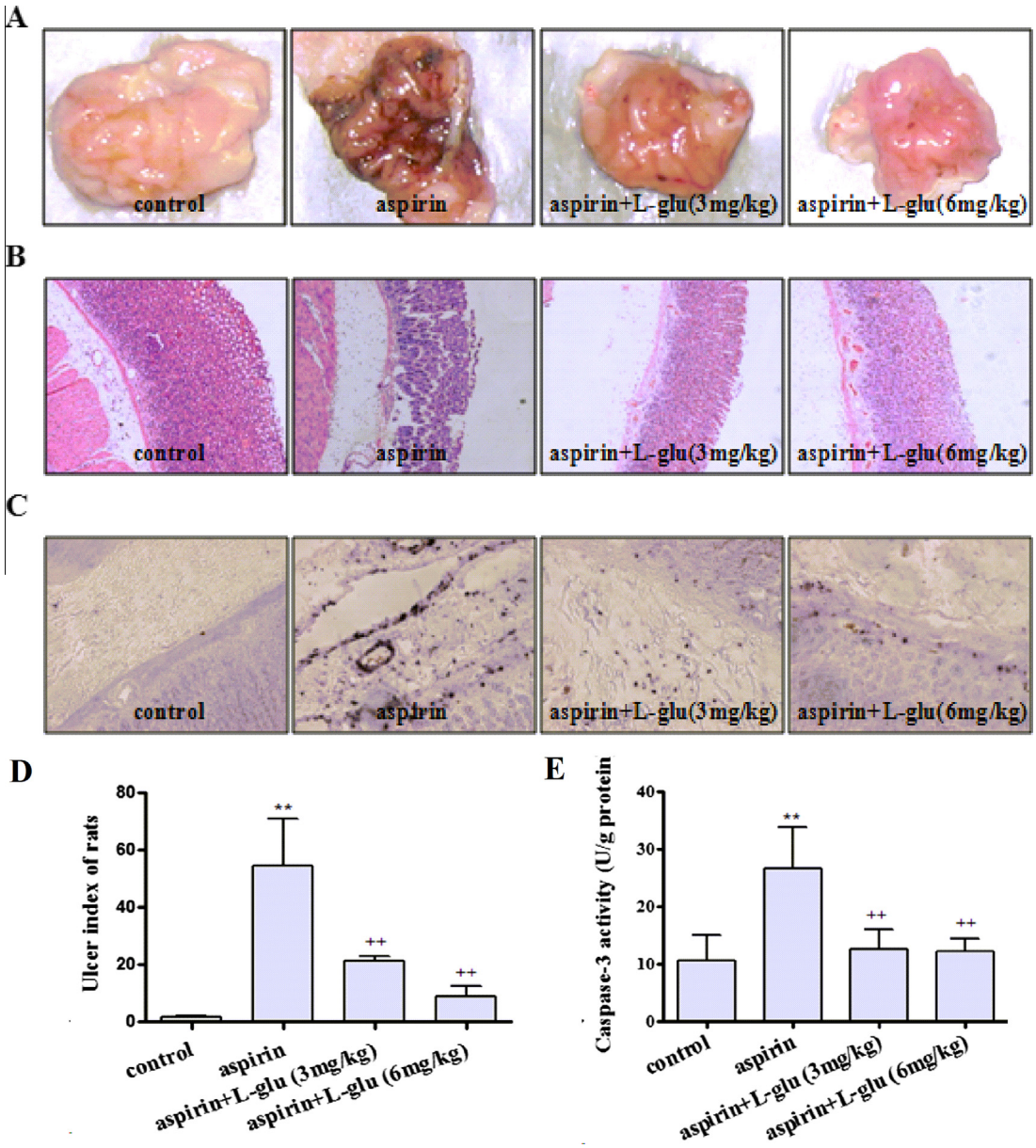

Fig. 2. Glutamate attenuates acute gastric mucosa injury and gastric apoptosis induced by aspirin in rats. (A) Appearance of gastric mucosa in rats; (B) HE staining of gastro (×100); (C) TUNEL staining of gastro in rats (×100); (D) quantification of A as ulcer index (UI), ulcer index is counted by GUTH ($n = 8$). (E) caspase-3 activity of gastro in rats. All data are expressed as mean ± SD ($n = 8$). All data are expressed as mean ± SD $^{**}P < 0.01$ versus control, $^{++}P < 0.01$ versus aspirin. L-glu is L-glutamate.

1.4. Cell culture

Human gastric epithelial immortalized cell line (GES-1) was cultured in Dulbecco modified Eagle medium, containing 10% fetal bovine serum, under the conditions of 5% CO2/95% air and 37 °C. After overnight incubation, the medium was replaced with fresh medium. Cells were treated with aspirin (10^{-6}–10^{-3} M) and L-glutamate (10^{-7}–10^{-6} M) in a dose range.

1.5. HE staining and TUNEL staining

Briefly, gastric tissues were fixed in 4% paraformaldehyde and embedded in paraffin and then cut into 5 μm sections. The slices were stained with hematoxylin and eosin for morphometric analysis and were examined with an Olympus microscope (Olympus Corporation, Tokyo, Japan). Cell death by apoptosis in gastro was evaluated by measurement of oligonucleosomal DNA fragments by a histochemical terminal deoxynucleotidyl transferase (TdT) TUNEL-like staining. paraffin-embedded sections were permeabilized with protease K (2 mg/ml) in 10 mM Tris, pH 8.0, at room temperature for 20 min. Endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 5 min. Sections were incubated with a reaction buffer composed by biotin-dCTP and unlabeled dCTP and TdT enzyme in a humidified chamber at 37 °C. In this assay, TdT binds to exposed 3′-OH ends of DNA fragments and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides were then detected using a streptavidin–horseradish peroxidase conjugate and diaminobenzidine.

1.6. Hoechst 33258 staining

To evaluate the apoptosis of cells, the method of Hoechst staining was applied. The GES-1 cells were fixed and stained with Hoechst 33258 according to the manual of Hoechst staining kit

(Beyotime, Shanghai, China). Stained cells were washed twice with PBS and imaged under a fluorescent microscope (excitation, 350 nm; emission, 460 nm). The number of apoptosis cells was presented as percentage of the total cells.

1.7. Caspase-3 activity assay

The activity of caspase-3 of GES-1 cells and gastric tissue were determined using the Caspase-3 activity kit (Beyotime, Shanghai, China). Cell lysates were prepared after their respective treatment with various designated treatments. Assays were performed on 96-well microtitre plates by incubating 10 μl protein of cell lysate per sample in 80 μl reaction buffer (1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM Nad and 10% glycerol) containing 10 μl caspase-3 substrate (Ac-DEVD-pNA) (2 mM). Lysates were incubated at 37 °C for 4 h. Samples were measured with an ELISA reader at an absorbance of 405 nm. The detail analysis procedure was described in the manufacturer's protocol.

1.8. LDH assay

One milliliter of cells at a density of 5×10^5 cells/ml were seeded in each well of 12-well plates and grown for 48 h before drugs exposure. The cells were washed with PBS two times and dosed with different concentrations of L-glutamate and aspirin in DMEM medium containing 1% FBS. After 24 h exposure, the 12-well plates were shaken briefly to homogenize the released LDH in the cell culture medium and the medium was transferred to microcentrifuge tubes and were centrifuged at 12,000×*g* and 4 °C for 15 min. One hundred microliters (100 μl) of each sample was added to the substrate solution and the absorbance at 340 nm was measured using a spectrophotometer.

1.9. Cell viability assay

The cell viability assay was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction assay which was performed to determine the cytotoxic effect of the aspirin and L-glutamate at various concentrations. Briefly, the GES-1 cells were plated onto 96-well flat bottom culture plates before various concentrations of drugs exposure. All cultures were exposure to drugs for 24 h at 37 °C in a humidified incubator. After 24 h of incubation, 10 μl of MTT (5 mg/ml in PBS) was added to each well, and the plate was incubated for a further 4 h at 37 °C. The resulting formazan was dissolved in 100 μl of DMSO with gentle shaking at 37 °C, and absorbance was measured at 595 nm with an ELISA reader. The results were given as the mean of three independent experiments.

1.10. Determination of glutamate release

To assay the releasing of glutamate, we measured glutamate concentration in gastric secretion and extracellular medium. One hundred microliters (100 μl) of each sample was added to the substrate solution and the absorbance at 340 nm was measured using a spectrophotometer.

1.11. Real-time PCR analysis

Real-time PCR was performed to quantify mRNA level of xCT and 4F2hc mRNA. Total RNA was extracted from gastric tissues and GES-1 cells by using TRIzol reagent (TakaRa, Dalian, China) and 500 ng RNA from each sample was subjected to reverse transcription reaction using the PrimeScript reverse transcription regent Kit (TakaRa, Dalian, China). The concentration and purity of eluted RNA was determined spectrophotometrically (O.D. 260/280 ratio between 1.8 and 2.2). Quantitative analysis of mRNA expression was performed by using the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR® Premix Ex Taq™ (TakaRa, Dalian, China). PCR cycling conditions were an initial incubation at 95 °C for 15 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 31 s. Generated cDNA was amplified by quantitative real-time PCR with specific forward and reverse primers for human-xCT (F:5′-gtggcagtgacctttctgag-3′, R:5′-cccatacccaccatcacacc-3′), human-4F2hc (F:5′-gctccctcctttccttgttcc-3′, R:5′-cacatccccaaagttaagcactac-3′), rat-xCT (F:5′-cctctgttcatcccagcatta-3′, R:5′-cccagtcaaggtgataaggaag-3′), Human-β-actin (F:5′-gcaccacaccttctacaatga-3′, R:5′-gtcatcttctcgcggttggc-3′). Rat-β-actin (F:5′-tgtcaccaactgggacgata-3′, R:5′-accctcatagatgggcacag-3′). Data analysis was performed by comparative Ct method using the ABI software. β-actin were used to normalize the expression of mRNA.

1.12. Statistical analysis

The results were presented as mean ± standard (SD). Statistical analysis was performed by ANOVA followed by the Newman–Student–Keuls test for multiple comparisons. A value of $P < 0.05$ was considered significant.

2. Results

2.1. Effect of aspirin on glutamate level and mRNA expression of cystine/glutamate transporter in rats

The mRNA expression of xCT in stomach was detected with real-time PCR, which was significantly down-regulated after aspirin administration (Fig. 1A). A similar result was seen in human gastric epithelial immortalized cell line (GES-1) (Fig. 1C). In contrast, 4F2hc mRNA was equally expressed in both control and aspirin treated group (Fig. 1D). We also detected the concentration of glutamate in gastric secretion and extracellular medium, which reflects the activity of cystine/glutamate transporter. As shown in Fig. 1B and E, Glutamate concentration was significantly reduced after aspirin administration compared to control groups.

2.2. Effect of aspirin on acute gastric mucosa injury in rats

We analyzed the gastric mucosal ulcer areas and histological changes in normal and experimental animals. Rats treated with aspirin (aspirin group) showed predominant mucosal hyperemia and hemorrhagic lesions with edema covering the total glandular area of the stomach and an increased ulcer index (Fig. 2A and D). HE staining showed gastric mucosal damage with dilation and exfoliation of gastric epithelial cells and disruption of mucosal layer (Fig. 2B). In agreement with the results of morphology assay, aspirin treatment also induced apoptosis in gastric mucosa with a tendency toward increasing the number of TUNEL-positive cells and up-regulated caspase-3 activity (Fig. 2C and E).

2.3. Effect of aspirin on cell viability and apoptosis in GES-1

We conducted the experiments on human gastric epithelial immortalized cell line (GES-1). The cell viability was dose-dependently and time-dependently reduced by aspirin (Fig. 3A). The concentration of aspirin that reduced cell viability 50% compared to the control was 1 mM for 24 h. We also found aspirin-induced, significant cell apoptosis by Hoechst staining (Fig. 4A).

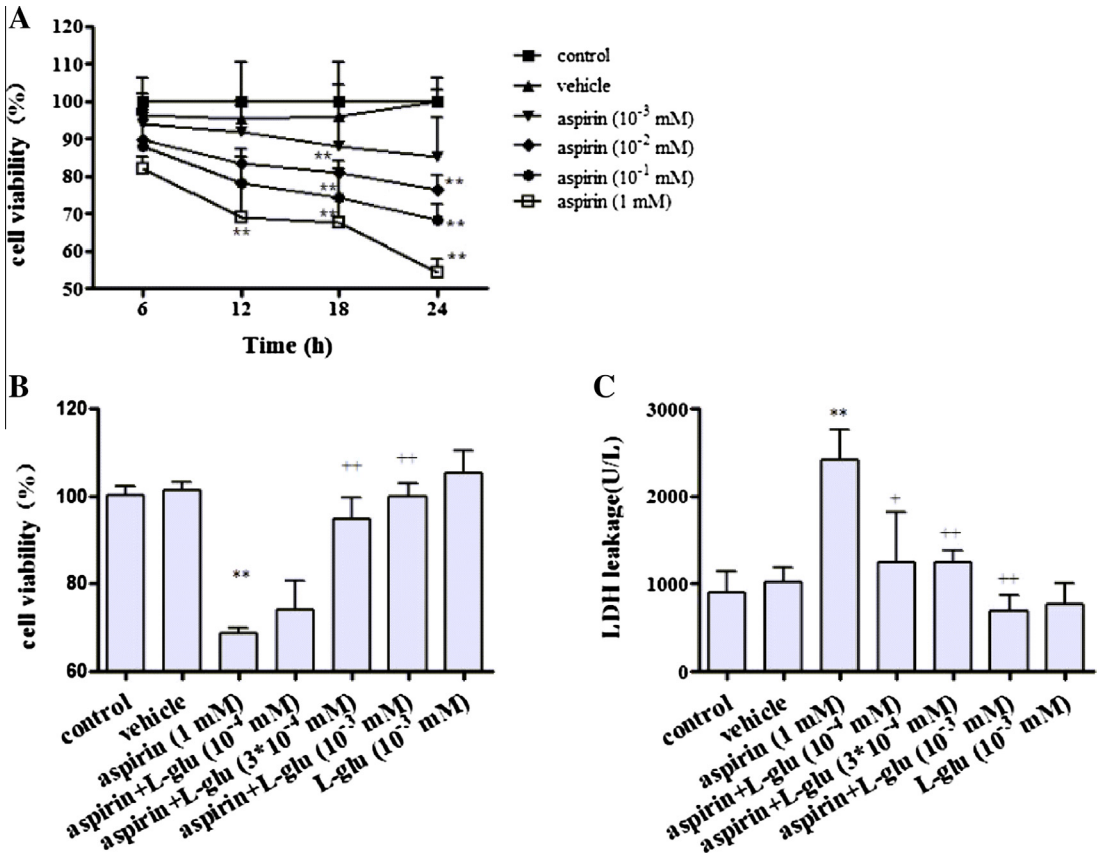

Fig. 3. L-Glutamate improved reduction of cell viability induced by aspirin in GES-1. (A) Time-dependent and dose-dependent effect of aspirin on cell viability assayed by MTT; (B) cell viability induced by aspirin and L-glutamate in GES-1; (C) LDH leakage determination in GES-1. All data are expressed as mean ± SD ($n = 3$). $^{**}P < 0.01$ versus control, $^{++}P < 0.01$ versus aspirin. L-glu is L-glutamate. GES-1 is human gastric epithelial cells, LDH is lactate dehydrogenase.

2.4. Effect of L-glutamate on aspirin-induced gastrotoxicity in rats

Exposure to aspirin for 6 h caused acute gastric ulcer measured as significant increases in UI and HE staining, these effects were reversed by treatment with exogenous L-glutamate (Fig. 2). Compared to aspirin group, rats pre-treated with L-glutamate at the doses of 3 or 6 mg/kg (L-glu (L) group, L-glu (H) group) had smaller mucosal ulcer areas, lower levels of hemorrhagic lesions (Fig. 2A and D) and improved morphological tissues(Fig. 2B). L-Glutamate treatment also inhibited the apoptosis and caspase-3 activity induced by aspirin in gastric mucosa(Fig. 2C and E).

2.5. Effect of L-glutamate on reduction of cell viability by aspirin in GES-1

In order to assess the direct protective effect of L-glutamate in vitro, we pretreated GES-1 with L-glutamate before aspirin incubation. MTT assays showed that L-glutamate at 0.3 μM and 1 μM significantly attenuated damage (approximately 20%) induced by aspirin. Lactate dehydrogenase (LDH), a marker of cell injury in extracellular media, was down-regulated by pre-treatment of glutamate which means a protective effect of glutamate (Fig. 3B and C).

2.6. Effect of L-glutamate on aspirin-induced apoptosis in GES-1

Meanwhile, according to the result of Hoechst staining, we found that aspirin-induced apoptosis was significantly decreased dose-dependently and time-dependently by L-glutamate (Fig. 4A and B). Using caspase-3 activity as a marker of apoptosis also showed the same conclusion (Fig. 4C), which suggests an inhibitory role of glutamate on aspirin-induced apoptosis in GES-1.

3. Discussion

Aspirin was initially isolated in 1897. Since then, numerous researches had revealed a wide pharmaceutical effect of aspirin and made it one of the most widely used medications. Now aspirin is used in treatment of pain, headache, fever and inflammatory diseases such as rheumatoid arthritis and pericarditis. However, clinical practices have found that aspirin causes serious gastrointestinal ulcers, especially in larger doses [16]. In the present acute gastric ulcer rat model induced by aspirin, significant gastric mucosa lesion including bleeding was observed. TUNEL staining indicated enhanced apoptosis in gastric mucosa. In vitro experiment also confirmed that similar damage was caused by aspirin on gastric epithelial cells. Increased apoptosis of gastric epithelial cells seems a major hallmark of gastric injury [17].

The pathogenesis of aspirin-induced gastric mucosa injury has not been fully established. It has been documented that alteration of multiple endogenous active substances such as prostaglandin, CGRP [18,19], nitric oxide [20] and leukotriene are involved in the progress. Glutamate is one of the most abundant neurotransmitters in the brain. Lots of studies have indicated that glutamate plays a key role in CNS [21,11,22]. Interestingly, subsequent research also revealed an important role of glutamate in peripheral tissues where glutamate usually acts as an extracellular signal mediator. Several studies have reported that glutamate plays a special role in regulation of gastrointestinal function [23–25]. Recently, studies revealed a relationship between aspirin and the

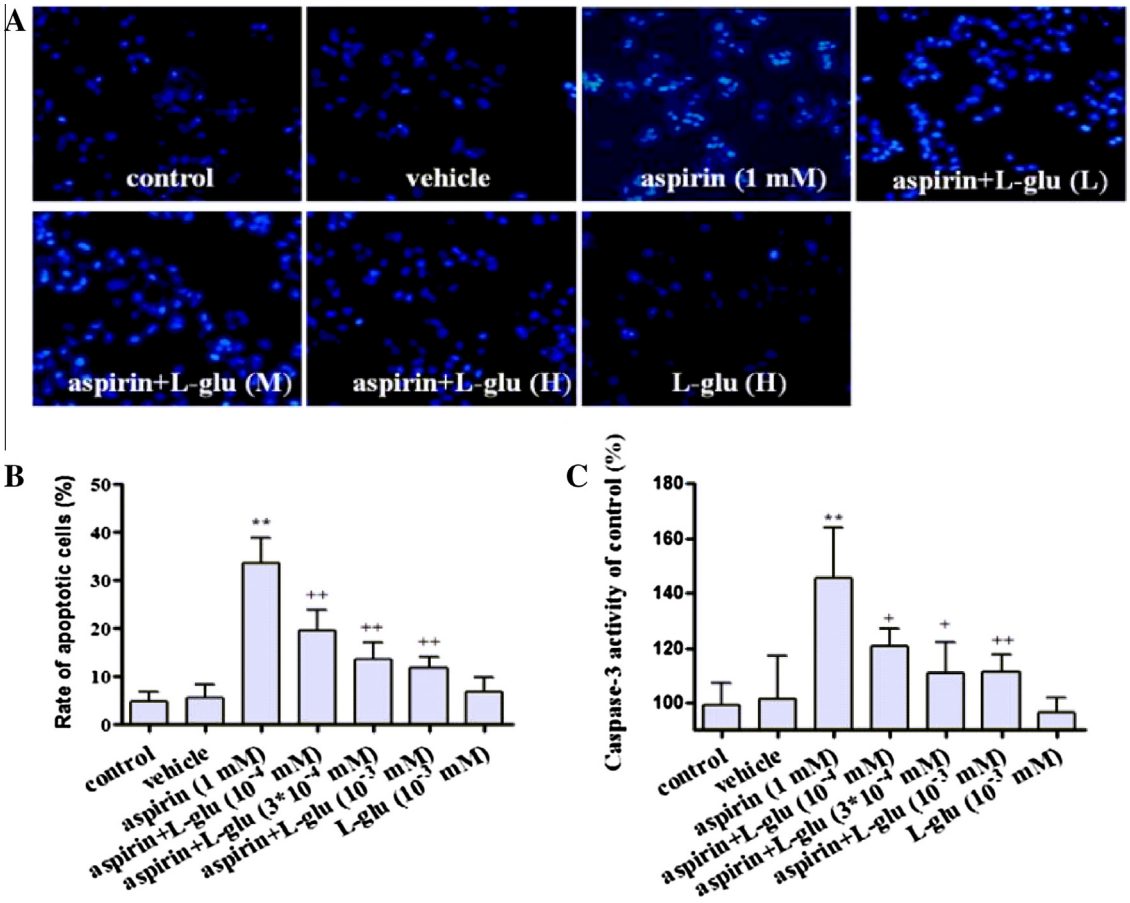

Fig. 4. L-Glutamate inhibited apoptosis induced by aspirin in GES-1. (A) Hoechst staining of GES-1; (B) statistical chart for A; (C) caspase-3 activity of GES-1. All data are expressed as mean ± SD ($n = 3$). $^{**}P < 0.01$ versus control, $^{+}P < 0.05$, $^{++}P < 0.01$ versus aspirin. L-glu is L-glutamate. GES-1 is human gastric epithelial cells.

activity of glutamate transporter. For example, aspirin is able to inhibit the release of glutamate and hence exerts a protective effect in CNS [11]. In our study, the results showed that aspirin also inhibited release of glutamate from gastric mucosa and cultured cells, accompanied with a damage of gastric mucosa. These results suggest that aspirin-induced acute gastric mucosa injury may involve the glutamate pathway.

The activity of cystine/glutamate transporter (consisting of 4F2hc and xCT), designated system Xc^-, is thought to be essential for maintaining intracellular glutathione levels and the extracellular cystine/cysteine redox balance. When extracellular cystine is taken up by cells via Xc^- system, intracellular glutamate will be released into the extracellular space at a ratio of 1:1. xCT is thought as the substrate-specific subunit of system Xc^-. Research also demonstrated that xCT can be strongly induced by various stimuli including oxidative stress, which suggests that xCT belongs to the adaptive cellular defense systems. In the present study, aspirin treatment significantly down-regulated the expression of xCT both in vivo and in vitro, which gave us a clue that xCT but not 4F2hc seemed to be the major response subunit adapting to aspirin stimulation.

In order to further establish that the inhibitory effect of aspirin on glutamate release is an important factor of gastric mucosa injury, the protective effect of exogenous glutamate on aspirin-induced gastric mucosa injury was investigated. The results demonstrated that exogenous glutamate significantly reduced damages of gastric mucosa due with aspirin in vivo. Similarly, glutamate treatment markedly attenuated cell injury and apoptosis in cultured human gastric epithelial cells. Again these findings supported the hypothesis that aspirin-induced acute gastric mucosa injury is associated with reduction of glutamate release via inhibition of cystine/glutamate transporter.

Acknowledgments

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