ORIGINAL ARTICLES

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The activation of high mobility group Box1 and toll-like receptor 4 is involved in clopidogrel-induced gastric injury through p38 MAPK

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Clopidogrel-induced gastric injury is an important clinical problem. However, the exact mechanism was still unclarified. This study aimed to investigate the role of high mobility group box protein 1 (HMGB1) and the toll-like receptor 4 (TLR4) pathway in normal human gastric epithelial (GES-1) cells under clopidogrel exposure. Morphological alterations of the gastric epithelial cells were observed under a microscope. A laser scanning confocal microscope was used to determine the distribution of HMGB1 and TLR4 in clopidogrel-induced injury. MTT was used to compare the viability of GES-1 cell among the pretreated Cli-095 (TLR4 inhibitor) group, pretreated ethyl pyruvate (HMGB1 inhibitor) group, clopidogrel group, and control group. Moreover, expression of the HMGB1, TLR4, tight junction (TJ) proteins occludin and the phosphorylation of the mitogen-activated protein kinases (MAPK) p38 were examined by western blot. We found the expression of HMGB1 and TLR4 in the cytoplasm increased after clopidogrel administration. Besides, inhibited TLR4, which is a receptor of HMGB1, prevented the injury and occludin reducing caused by clopidogrel challenge. Furthermore, blocking HMGB1 or TLR4 activity by ethyl pyruvate (HMGB1 inhibitor) or cli-095(TLR4 inhibitor) can partially diminish the activation of p38MAPK. Gastric mucosal damages observed by clopidogrel challenge are associated with HMGB1, TLR4, through p38MAPK signal pathway.

1. Introduction

Patients with acute coronary syndrome or those undergoing percutaneous coronary intervention and stent implantation significantly benefit from the use of clopidogrel alone or in combination with aspirin (Xie et al. 2011). However, it is documented that pretreatment with a loading dose of clopidogrel alone or in combination with aspirin is greatly improving patients' survival but is as associated with gastrointestinal (GI) complications, such as upper GI haemorrhage and recurrent gastric ulcer (Chan et al. 2005; Taha et al. 2006; Ziegelin et al. 2006; Shin et al. 2012; Takayama et al. 2012; Shmulevich et al. 2011; Uotani et al. 2012). Because dual antiplatelet therapy plays an important role in the treatment of cardiac disorders or peripheral arterial diseases, it is important to clarify the mechanism underlying drug-induced gastric bleeding and ulceration.

We previously demonstrated that clopidogrel-induced gastric injury is related to p38 mitogen-activated protein kinases (MAPK) activation and decreased expression of tight junction (TJ) proteins. MAPK which is associated with epithelial barrier function is a family of serine-threonine kinases that are responsible for intracellular signaling pathways (Wu et al. 2013). p38 is a member of the MAPK family, which plays a crucial role in clopidogrel-induced gastric injury. A single layer of epithelial cells throughout the whole GI tract is crucial for maintaining gastric mucosal immune homeostasis and resist to hazardous materials, such as strong acids and the protease pepsin. Loss of the integrity of epithelium is associated with gastric inflammation and even tumor development (Agarwal et al. 2009; Hayashi et al. 2012). Adherens junctions, desmosomes, and TJ proteins form the TJ complex, which is primarily responsible for epithelial barrier function. Among them, TJ proteins comprising occludin, the claudin family and junction adhesion molecule (JAM) is at the top of this bulwark, and plays a pivotal role in sealing the intercellular space in epithelial and endothelial cellular sheets (Hayashi et al. 2012; Chen et al. 2012; Tuskida et al. 2001). Among the multiple components of TJ, occludin and ZO-1 are crucial to assemble TJ. Occludin is a common component of the TJ in most types of epithelia without tissue and species specificity (Zhang and Fisher 2012). So occludin is a reliable structural and functional marker to evaluate the damage caused by clopidogrel.

Recent studies have demonstrated an association of GI mucosal injury with Toll-like receptor4 (TLR4) (Watanabe et al. 2008; Nadatani et al. 2012; Zhang et al. 2013; Ye et al. 2013). The Toll-like receptor (TLR) family plays an important role in innate immune responses against microbial pathogens, as well as in the subsequent induction of adaptive immune responses (Watanabe et al. 2008). TLR4 which recognizes pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) is a member of the TLR family. One of the most prevalent DAMPs is the high mobility group box protein 1 (HMGB1). HMGB1 ubiquitously exists in all eukaryotic cells, stabilizing nucleosome formation and regulating transcription of many genes in the nucleus. When it comes to the events such as trauma, ischemia/reperfusion, infarction (in myocardium) or infection, HMGB1 acts as an alarmin released into the extracellular environment (Nadatani et al. 2012; Ye et al. 2013). Nadatani et al. (2012) suggested that nonsteroidal anti-inflammatory drugs (NSAIDs) induced small intestinal damage was associated with HMGB1, TLR4. TLR4 has been shown to be expressed in many tissues, including gastric endothelial cells (Wang et al. 2011). Thus it is suggested that HMGB1, TLR4 activation may be involved in clopidogrel-induced gastric injury. HMGB1, TLR4 may be the upstream signaling pathway above p38MAPK. Thus, in this study we aimed to investigate the role of HMGB1, TLR4 in the pathogenesis of clopidogrel-induced gastric injury.

2. Investigations and results

2.1. Morphological alterations of the gastric epithelial cell line in response to HMGB1 inhibitor and TLR4 inhibitor after clopidogrel exposure

Morphological alterations of the gastric epithelial cell were observed under a microscope. Compared with control group, the number of GES-1 cells in the CL group was significantly reduced. Although, in the ethyl pyruvate preconditioned group, the number of GES-1 cells was still not as high as in the C group, so obviously, the EP group improved the viability of GES-1 cells. When it comes to the Cli-095 group, the number of GES-1 cell was higher than in the EP group, but still less than in the C group. In general, clopidogrel decreased the number of GES-1 cells, inducing serious damage. However, blocking the release of HMGB1 or TLR4 can relieve the damages caused by clopidogrel (Fig. 1).

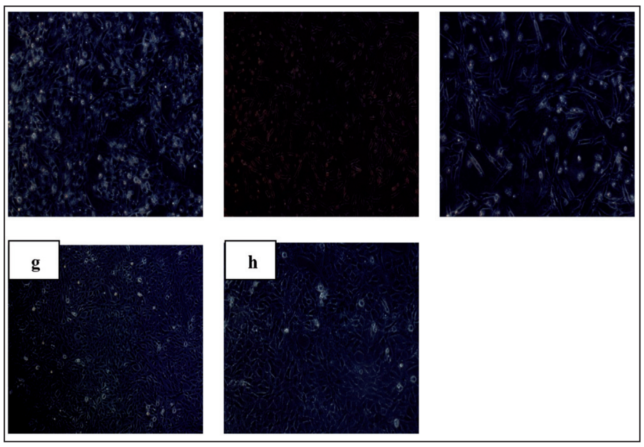


Fig. 1: Morphological alterations of GES-1,which were observed by microscope (Magn. Fig. 1 a, c, e, g 10x, Fig. 1 b, d, f, h 20 x). Fig. 1 a, b are pictures taken from Cli-095 group. Figs. 1c, d are pictures taken from EP group. Figs. 1e, f are pictures taken from CL group. Figs. 1g, h are pictures taken from C group.

2.2. Expression of HMGB1, TLR4 after clopidogrel administration

HMGB1 localization was mostly limited to inside the nuclei of epithelial cells in the control group. This is consistent with the fact that HMGB1 is a highly conserved nuclear in nuclear (Bustin 1999). Compared with the control group, prominent cytoplasmic staining of HMGB1 in epithelial cells was observed after clopidogrel exposure via Laser scanning confocal microscope. Because necrotic or damaged cells, but not apoptotic cells, passively release HMGB1 (Scaffidi et al. 2002), the results suggest that clopidogrel can damage GES-1 cells and induces GES-1 cell necrosis, leading HMGB1 release into the cytoplasm. As to TLR4, clopidogrel increased the activity of TLR4. Immunofluorescence staining **was** significantly higher in the GES-1 cells after clopidogrel administration, suggesting that TLR4 is associated with clopidogrel-induced damages (Fig. 2).

2.3. Effects of HMGB1, TLR4 on GES-1 viability during gastric injury induced by clopidogrel

Our previous study has shown that clopidogrel induce GES-1 cells apoptosis in a dose- and time-dependent manner (Wu et al. 2013). In the present study, MTT examination revealed that clopidogrel significantly reduced GES-1cell survival rate. The viability of GES-1cell was raised when pretreated Cli-095 (TLR4 inhibitor) or ethyl pyruvate (HMGB1 inhibitor), suggesting that a

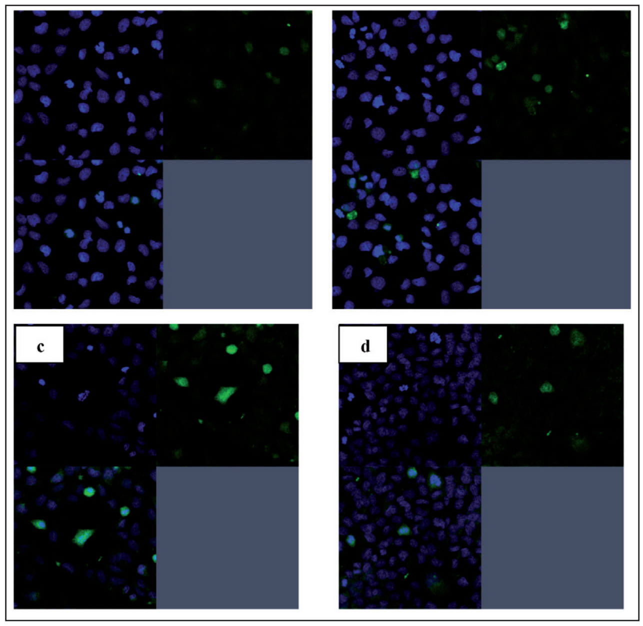


Fig. 2: Immunofluorescence staining of the locations of HMGB1 and TLR4. Figs. 2a and b are targeted on HMGB1. Fig. 2a is taken after clopidogrel exposure, Fig. 2b is taken before addition of clopidogrel. Fluorescence intensity is higher in the cell nucleus before clopidogrel was added, then transfer to the cytoplasm when clopidogrel was added. Figs. c and d are targeted onTLR4. Fig. 2c is taken after clopidogrel exposuer, Fig. 2d is taken before the addition of clopidogrel. The fluorescence intensity increased after clopidogrel was added.

decreased release of TLR4 or HMGB1 can improve the viability of GES-1cells. This MTT results together with what we know from Immunofluorescence staining and the morphological alterations of GES-1cells, strongly imply that clopidogrel causes marked damages of the gastric mucosa, while inhibiting HMGB1 or TLR4 would relieve the destruction mediated by clopidogrel, at least in part. Moreover, viability of GES-1cells pretreated with Cli-095 is higher than after pretreatment with ethyl pyruvate. This may be due to TLR4 as downstream factor of HMGB1, which plays a more important role, or some other upstream factor will act synergistically with HMGB1 to stimulate TLR4 and its downstream signal pathway (Fig. 3).

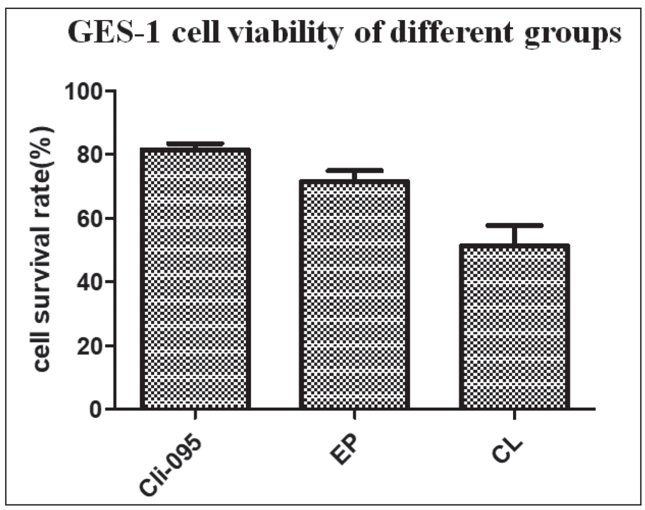
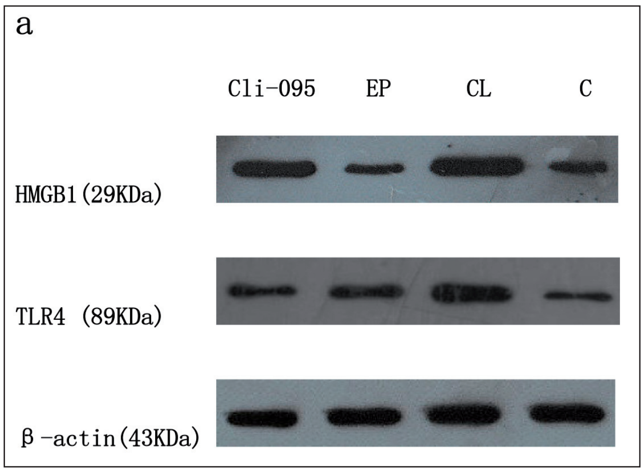
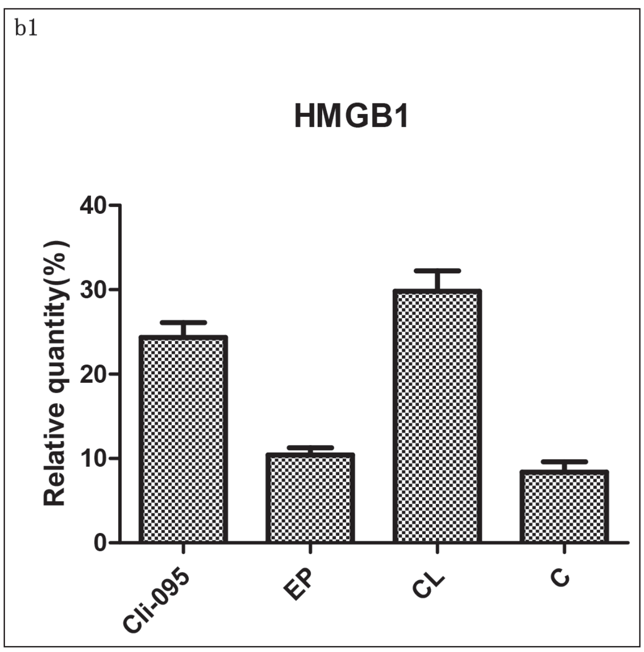


Fig. 3: Viability and proliferation of the GES-1 cells of each group. Cell viability was determined as a percentage using the following equation: (ODA570 nm in experimental group/ODA570 nm in normal control group) ×100%. All experiments were repeated at least three times. Cli-095 represents the Cli-095 preconditioning group, EP means the Ethyl pyruvate preconditioning group, CL means clopidogrel group. $p<0.05$vs. vehicle control.

2.4. Role of HMGB1, TLR4 in clopidogrel-induced mucosa damages

Occludin is one of the most important TJ proteins regulating the TJ barrier function and it is known that clopidogrel impaires gastric mucosal barrier properties and TJ organization. Hence, occludin would provide a useful clue about the mechanism of clopidogrel-induced gastropathy (Wu et al. 2013; Chen et al. 2012; Zhang and Fisher 2012). Therefore, we used the expression quantity of occludin to evaluate the direct effects clopidogrel on GES-1cells. In this study, compared with control group, the quantity of occludin decreased sharply in the CL group as determined by Western blot analysis, suggesting that clopidogrel badly impaired TJ barrier function. However, the effects were attenuated by pretreatment with an TLR4 inhibitor. Cli-095, likewise, when pretreated with ethyl pyruvate, a HMGB1 inhibitor, increased the quantity of occludin, compared with the CL group. In addition, blocking the release of TLR4 seemed more effective to raise the quantity of occludin and to reverse the damages caused by clopidogrel. In short, HMGB1 and TLR4 played a crucial role in gastric mucosa damages, and the HMGB1- TLR4 signal pathway is involved in gastric barrier dysfunction after clopidogrel exposure (Fig. 4).





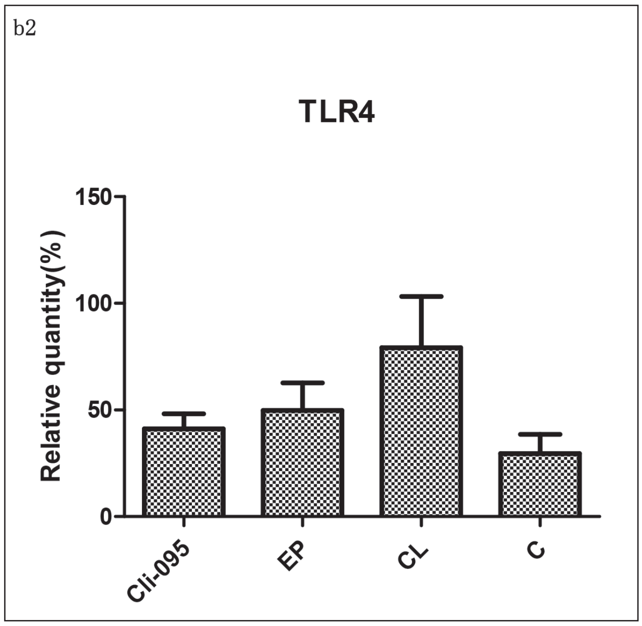


Fig. 4: Expression of HMGB1, TLR4 through western blot. The expression of HMGB1, TLR4 increased after clopidogrel administration, All experiments were repeated at least three times. $p<0.05$ vs. vehicle control.

2.5. Activation of p38MAPK signal transduction in clopidogrel-induced GES-1cell injury

Phosphorylated p38 is the activated form of p38MAPK, which is a clue to clopidogrel-induced GES-1cell injury. Therefore, we used the expression quantity of phospho-p38 to assess clopidogrel-induced inflammation, and also to investigate the relationship between HMGB1, TLR4 and p38MAPK. Western blot analysis showed that clopidogrel increased the quantity of phospho-p38 as expected. Cli-095 (TLR4 inhibitor) or ethyl pyruvate (HMGB1 inhibitor) can prevent the increase of phospho-p38. This demonstrated that the activation of p38MAPK signal transduction is involved in the HMGB1- TLR4 signal pathway.

3. Discussion

Various factors such as increased oxidative stress, induction of cell apoptosis, increased release of pro-inflammatory cytokines, activation of hormonal stress response could affect the internal environment of the human body. The interplays between the physiological changes associated with tissue injury and the ensuing inflammation which involve cell death and apoptosis of tissue cells are complicated (Fink et al. 2018; Erwig and Henson 2007). To identify which factor is associated with a certain related symptom, the *in vitro* cell study is an appropriate approach, because confounding factors would be minimized or avoided (Wu et al. 2013). In the present study, we inhibited HMGB1 release by ethyl pyruvate decreased clopidogrel-induced gastric injury by increasing occluding expression and improved cell survival rate at the same time. Furthermore, inhibition of TLR4, which is a receptor of HMGB1, prevented injury and occludin expression. These findings suggest that HMGB1 and TLR4 play a critical role in clopidogrel-induced gastric injury.

Many reports indicate the importance of HMGB1 and TLR4 related tissue injuries. Ye et al. (2013) used animal models to demonstrate that Toll-Like Receptor 4 is associated with a susceptibility to ethanol-induced gastric mucosal injury. Increased expression of HMGB1 may lead to increased secretion and binding to TLR4, and further aggravated the ethanol-induced gastric mucosal injury. Nadatani et al. (2013) showed that HMGB1 inhibits the gastric ulcer healing process, which acts through TLR4 and advanced glycation end products (RAGE) to induce excessive inflammatory responses. Their previous study (Nadatani 2012) also demonstrated that HMGB1 and TLR4 are associated with indomethacin-induced small intestinal damage. All these results are consistent with our findings. In our study, the results of immunofluorescence give us a direct visual impression of the fact that the expression of HMGB1 and TLR4 in the cytoplasm increased after clopidogrel administration. Also with other approaches, it can be shown that HMGB1 and TLR4 are involved in clopidogrel-induced gastric injury.

Several reports have indicated an activation of MAPKs through the HMGB1-TLR4 signaling pathway. It was found that HMGB1 acts in synergy with lipopolysaccharide (LPS) to upregulate TLR4 expression on the surface of synovialibroblasts (SF) in rheumatoid arthritis (RA) *via* p38 MAPK signaling pathways (He et al. 2013). Tsung et al. (2005) implicated that HMGB1 stimulates the phosphorylation of p38 (pp38) and JNK in I-R liver injury through the activation of TLR4 signaling. Moreover, some studies demonstrated the relationship between HMGB1, TLR4 and epithelial function. Sappington et al. (2002) used mice models to find that HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in a time- and dose-dependent manner. Yang et al. (2009) also used animal models to prove that bile HMGB1 mediates LPS-induced gut barrier dysfunction and regulates mucosal permeability and bacterial translocation. Dai et al. (2010) found that HMGB1 inhibits enterocyte migration *via* the activation of TLR4. Consistent with Dai, Rousseau et al. (2013) showed that LPS can increase the migratory ability of human esophageal cancer cells by increasing their adhesive properties through TLR4, p38 signaling. In the present study, the expression of pp38 reduced while the level of occludin raised when we inhibited HMGB1 release. The situation appeared again, when we use cli-095 to depress TLR4 release. This strongly suggests that the HMGB1-TLR4 signaling pathway is associated with p38MAPK, and epithelial barrier function.

HMGB1, a member of the high-mobility group protein superfamily is both a nuclear factor and a secreted protein. In the cell nucleus it acts as an architectural chromatin-binding factor that bends DNA and promotes protein assembly on specific DNA targets (Scaffidi et al. 2002). Once leaked or actively secreted into the extracellular environment, HMGB1 acts as an alarmin with potent proinflammatory properties. The extracellular HMGB1 may has two possible sources: passively released from necrotic cells or damaged cells and actively secretion from inflammatory cells such as activated monocytes and macrophages (Sacffidi et al. 2010; Nadatani et al. 2013; Andersson et al. 2000). In this study, HMGB1 was observed by immunofluorescence in the cytoplasm as well as the nucleus, suggesting that necrotic cells are the source of HMGB1in clopidogrel-induced gastric injury. Necrotic cells can release HMGB1, but apoptotic cells do not release HMGB1 even after undergoing secondary necrosis and partial autolysis (Scaffidi et al. 2002), according to this and our results from cell morphology under the microscope, we considered it plausible that HMGB1 activation is involved in clopidogrel-induced gastric epithelia injury. However, our previous study found that clopidogrel led to a marked induction of GES-1 cells apoptosis. And there was a significant difference in the apoptosis rate between vehicle control cells and cells treated with clopidogrel (Wu et al. 2013). This may suggest that clopidogrel can induce GES-1 cells necrosis, damage, and apoptosis as well, but only necrotic or damaged cells stimulate HMGB1, through the HMGB1-TLR4 signal pathway. TLR4 can be activated by either PAMPs, *H. pylori* infection for example, or DAMPs, like exogenous toxin injury. In our study, the expression of TLR4 increased as immunoreactivity was observed in the cytoplasm. Moreover, when we inhibited the release of HMGB1, the level of TLR4 reduced as well. These suggested that the injury clopidogrel caused, TLR4 is activated by HMGB1,which is a member of DAMPs. And HMGB1-TLR4 signaling pathway is involved in clopidogrel-induced gastric injury.

From this study, we found that clopidogrel can severely cause cell necrosis and suppress GES-1 cell viability. Attenuated expression of occludin after clopidogrel exposure was associated with impaired epithelial barrier integrity. The incomplete barrier may lead to increased permeability of gastric epithelial cells and increase the damage. However, these observed effects were partially abolished by cli-095 (a TLR4 inhibitor), and ethyl pyruvate (a HMGB1 inhibitor). Furthermore, it seems that cil-095 is more effective than ethyl pyruvate, maybe because TLR4 is the downstream signal factor, which plays a more direct role in this signal pathway. Or, another harm factor would stimulate TLR4

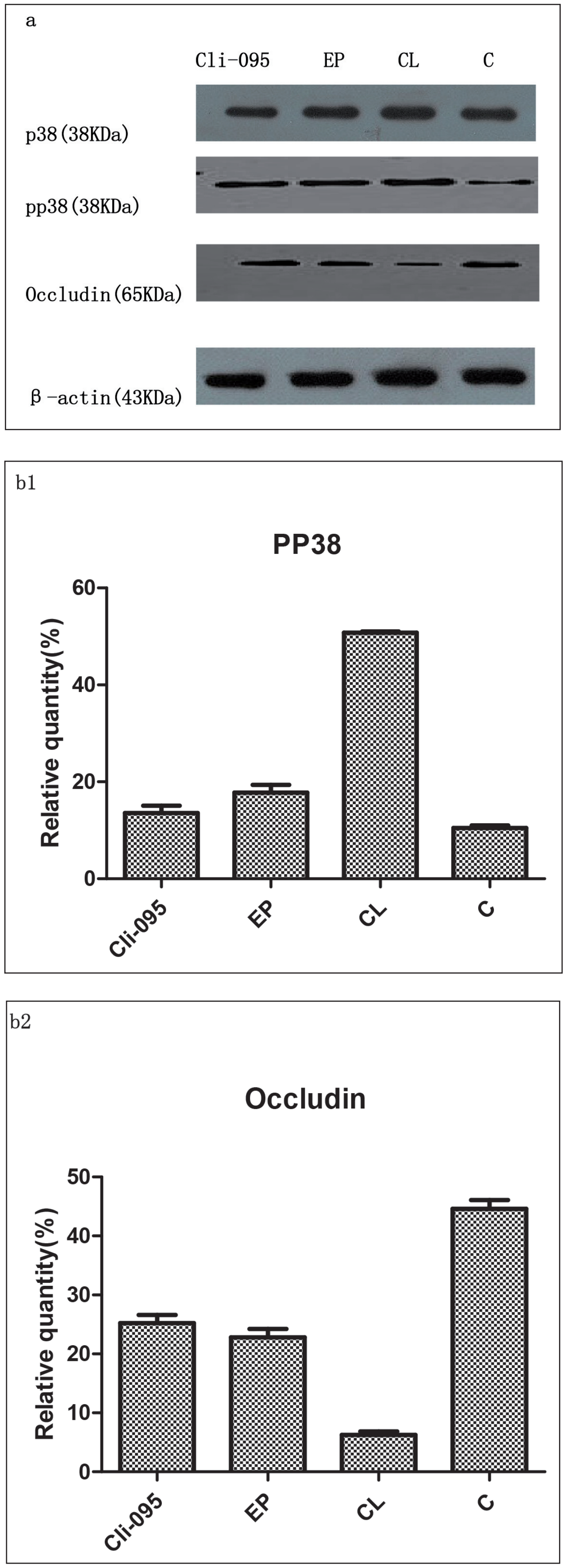


Fig. 5: Expression of PP38, occludin through western blot. The expression of PP38 increased after clopidogrel administration, while the expression of Occludin decreased after clopidogrel was added. Cli-095 or EP can partly reduce the decrease of occludin (Fig. 5b2) and relieve the increase of pp38 (Fig.5b1). All experiments were repeated at least three times. p<0.05vs. vehicle control.

signal pathway. It seems like TLR4 and its downstream pathway is the final signal pathway of clopidogrel mediated injury. Our previous study has demonstrated that clopidogrel induced phosphorylation of three important MAPKs p38, ERK, and JNK, but only the p38MAPK inhibitor SB-203580 attenuated increased permeability and disruption of the TJ induced by clopidogrel (Wu et al. 2013). Furthermore, HMGB1-TLR4 interaction stimulated phosphoMAPKs were observed in inflammatory cells after indomethacin exposure (Nadatani et al. 2012). In this study, blocking HMGB1 or TLR4 activity by ethyl pyruvate (HMGB1 inhibitor) or cli-095 (TLR4 inhibitor) can partially diminish the activation of p38MAPK. So we think that activation of HMGB1 and TLR4 is involved in clopidogrel-induced gastric injury through p38MAPK. There were some limitations to the present study worth further consideration. This study just observed direct effects of clopidogrel itself on a single layer of GES-1 cells *in vitro*. However, in the human body, clopidogrel forms its active metabolite in the liver exerting its antiplatelet effect. In addition, multiple genetic and nongenetic factors such as ethnicity, gender, increased age and BMI, can impact the pharmacological and clinical responses to clopidogrel (Xie et al. 2011). Therefore, in this study the gastric mucosal damage caused by clopidogrel is just the result of the direct toxic effect of the parent drug itself, rather than of its active metabolite, whereas the overall toxic effects *in vivo* should be the sum of parent drug and active metabolite (Wu et al. 2013).
At least three receptors (ie,TLR2, TLR4, and RAGE) have been reported to mediate the proinflammatory effects of HMGB1 (van Zoelen et al. 2009; Park et al. 2006). As TLR4 is associated with NSAID-induced damages (Nadatani et al. 2012), we just focus on TLR4. Therefore, further study can stress other receptors. Although some limitations exist, our findings should be valuable pieces of a big picture. Each pieces can be used to fit the puzzle derived from clinical practice, further risk prediction and future risk stratification (Xie et al. 2011).
Gastric mucosal damages observed by clopidogrel challenge are associated with HMGB1, TLR4, throgh p38MAPK signal pathway. Blocking HMGB1, TLR4 or inhibiting HMGB1-TLR4 signal pathways may be a useful therapeutic option against clopidogrel-induced injury.

4. Experimental

4.1. Materials

4.1.1. Drug

Clopidogrel (purity: 99.18 %; lot # NDS1305015) was purchased from Beijing Nordhuns Chemical Technology Com. Ltd., China. Dimethyl sulfoxide (DMSO) was added into the clopidogrel powder weighed. Finally a final concentration of clopidogrel of 0.5 mM was reached, the concentration of DMSO present in the working medium was restricted to be 0.1 % (v/v) based on preliminary results (data not shown). A working medium which contained 0.1 % DMSO was used as the vehicle controls throughout all other relevant cell studies (Wu et al. 2013). Cli-095, TLR4 signaling inhibitor, was purchased from invivogen (#09D03-MM). Ethyl pyruvate, which is an inhibitor of HMGB1 release, was purchased from Sigma-Aldrich. Rabbit polyclonal anti-HMGB1 (#3935), Rabbit polyclonal anti-p38MAPK(#8690) were purchased from Cell Signaling Technology. Beverley, MA, USA. Rabbit polyclonal anti-TLR4(sc-10741), Rabbit polyclonal anti-occludin(sc-5562) were purchased from Santa Cruz Biotechnology (USA).

4.1.2. Cell culture

The normal human gastric epithelial cell line (or called GES-1) was obtained from the Division of Gastroenterology, Department of Medicine, People's Hospital of Jiangsu Province, China, DMEM-HG (Hyclone, Logan,UT, USA) was supplemented with 10 % FBS and 1 % antibiotics. The culture medium was placed in a humid incubator with 95 % air and 5 % CO_2 at 37 °C and was changed every 48-72 h.

4.2. Methods

4.2.1. Morphological alteration of GES-1 under microscope

The complete medium was replaced with DMEM containing 1 % FBS one day before the experiment to synchronize the cells. The cells were randomly divided into 4 different groups:
Cell control group (C): cells treated with the medium containing 0.1% DMSO; Clopidogrel group (CL): cells treated with the medium containing 0.5 mM of clopidogrel; Cli-095 preconditioning group (Cli-095): cells treated with Cli-095 (1 μg/ml), incubated for 6 h at 37°C, then treated with the medium containing 0.5 mM of clopidogrel; Ethyl pyruvate preconditioning group (EP): cells treated with ethyl pyruvate (10 μg/ml), incubated for 6 h at 37 °C then treated with the medium containing 0.5 mM of clopidogrel.
Then the cells were cultured for 12 h, after that morphological alteration of GES-1 was observed under a miscroscope.

4.2.2. Immunofluorescence staining

The GES-1 cells were seeded in 4 cell imaging dedicated glass bottom culture dishes (35 mm *15 mm), two of them monolayers were treated with vehicle control the rest were treated with clopidogrel. Twelve hours later, immersed in 4 % paraformaldehyde fixation in 30 min or overnight, to improve the permeability of the cell, cells were washed with the PBS solution 3 min * 3, fixed with ethanol (30 min), immersed in 4 % paraformaldehyde fixed liquid at room temperature, fixed 20 min, PBS washed 3min * 3, used 3 % H_2O_2 methanol solution closed 10 min at room temperature. Used goat serum 50-100 μl, incubation for 20 min at room temperature. Adding primary antibody (1:200 dilution) 50-100 μl, 37 °C, wet box were incubated for 2 h. Adding FITC/TRITC (1:200 dilution) secondary antibodies 50-100 μl, 37 °C, dark incubation of 1 h and stained with DAPI (50-100 μl, 5 min). Slides were viewed using a laser scanning confocal microscope (IX51, Olympus, Japan), the results are shown in Fig. 2.

4.2.3. MTT assay

MTT assay was used to determine the viability and proliferation of the GES-1 cells of each group. The cells were seeded in 96-well plates at 5×10^3cells/well in 200 μL medium and cultured for 12 h to allow attachment, and divided into 5 groups, 4 of them as we told above, the last one was blank control group (PBS only). The cells were treated with MTT solution (5 mg/mL in 20 uL PBS) (KeyGEN Biotechnology, Nanjing, Jiangsu, China) and incubated for 4 h in the darkness at 37 °C. After removing the medium, the growth groups were replaced with DMSO (200 μL/well) to dissolve MTT-formazan crystals and maintained for 10 min. The blank control group (PBS only) was used for setting of zero point. Absorbance (A) of each sample was measured at 570 nm. Cell viability was determined as a percentage using the following equation: (ODA570nm in experimental group/ODA570 nm in normal control group) ×100%. All experiments were repeated at least three times.

4.2.4. Western blotting analysis

After the experiment, the cells were washed with cold PBS twice before the mixture of lysis buffer made by the protein extraction kit (KeyGEN Biotechnology, Nanjing, Jiangsu, China) was added to the flasks and placed on ice for 30 min to split the cells. The cell fragments were harvested and centrifuged for supernatant collection (total proteins). The protein concentrations were determined by bicinchoninic acid (BCA) assay. Lysates were boiled in sample buffer for 10 min and then the proteins were subjected to 10 % SDS-PAGE gels, and transferred to nitrocellulose (NC) membrane using a semi-dry electroblotting system. Skimmed milk (5 %) in Tris-buffered saline (pH 7.5)–Tween 20 for 2 h at room temperature was used to block the nonspecific antigen in the membrane. After incubation with appropriate primary antibody (1:1000) at 4 °C overnight, it was extensively washed with TBST buffer, and incubated with the suitable secondary antibody (1:2000) for 2h. At last developed by an enhanced chemiluminescence system (KeyGEN Biotechnology, Nanjing, Jiangsu, China) and captured on a light-sensitive imaging film. Protein expression was analyzed semi-quantitatively by using the Gel and Graph Digitizing System (Silk Scientific, Tustin, CA, USA). All experiments were done at least three times.

4.2.5. Data analysis and statistics

All data were described as mean±standard deviation (SD). One-way analysis of variance (ANOVA) was used to test the significance of the differences between the experimental groups by SPSS20.0. The results were analyzed with Fisher Student Neuman Keuls test. P-values less than 0.05 were considered statistically significant.

Conflicts of interest: None declared.

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