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The selective ASIC3 inhibitor APETx2 alleviates gastric mucosal lesion in the rat

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This study aimed to assess the *in vivo* efficacy of acid sensing ion channel 3 (ASIC3) inhibitor APETx2 to alleviate acute gastric mucosal lesion (AGML) in a rat model. Thirty-six male Wistar rats were divided randomly into three groups: control group, water immersion restraint stress (WIRS) group, and APETx2 treatment group (n = 12). AGML was induced by WIRS for 6 h, and 25 μg/kg APETx2 was injected intraperitoneally before the onset of stress. Intragastric pH, ulcer index (UI) and gastric histopathological changes were measured, ASIC3 expression in thoracic dorsal root ganglia (DRG) neurons was examined by immunohistochemistry, PCR and Western blot analysis. Compared with control group, WIRS group showed obvious gastric injury with increased UI score, decreased intragastric pH and increased ASIC3 expression in DRG neurons ($p < 0.05$). APETx2 treatment before WIRS significantly alleviated gastric mucosal injury, decreased UI score, decreased gastric acidity and reduced ASIC3 expression in thoracic DRG neurons ($p < 0.05$). In conclusion, ASIC3 expression in DRG neurons projecting to the stomach is positively correlated with gastric mucosal lesion and acidosis in WIRS model. ASIC3 inhibitor APETx2 could improve gastric acidosis and alleviate AGML.

1. Introduction

Acute gastric mucosal lesion (AGML) or stress ulceration indicates the diffuse lesions of the mucosal layer in the upper gastrointestinal tract, and is frequently associated with a high rate of morbidity and mortality in critically ill patients (Duerksen 2003). AGML is caused by a variety of factors, including hypovolemia, acidosis due to mucosal ischaemia (hypoxia), inflammatory mediators released at the site of injury and acid back-diffusion (Jia et al. 2007). However, the pathogenesis of AGML remains elusive.

Acid-sensitive ion channels especially acid-sensing ion channels (ASICs) function to regulate afferent neurons to modulate gastric acidosis (Holzer 2009). ASICs consist of sodium-selective ion channels which are activated by low extracellular pH (Waldmann et al. 1997). Among them, ASIC3 is the most sensitive to pH change. ASIC3 is abundantly expressed in dorsal root ganglia (DRG) neurons (Lingueglia 2007; Wemmie et al. 2006). A retrograde tracing study in rats indicated that 82% of DRG neurons projecting to the stomach demonstrated ASIC3 immunoreactivity (Schicho et al. 2004). Recent evidence suggests that ASIC3 is implicated in the control of pain associated with tissue acidosis after inflammation or injury (Karczewski et al. 2010; Yen et al. 2009). Experimental induction of gastric ulcers could change acidosis induced currents in the stomach-innervating neurons of DRG (Sugiura et al. 2005). Nonetheless, it remains unclear whether ASIC3 plays a role in stress induced gastric ulcer.

Table: Comparison of intragastric pH and UI values in three groups

Groups	Values of pH	Ulcer index
Control group	3.46 ± 0.31	0.0 ± 0.0
WIRS group	1.85 ± 0.20*	62.6 ± 5.4*
APETx2 treatment group	2.63 ± 0.47*Δ	18.5 ± 3.6*Δ

Data were expressed as means ± SEM (n = 12). *$p < 0.05$ versus control group, Δ $p < 0.05$ versus WIRS group.

Therefore, in this study we aimed to investigate the role of ASIC3 in stress gastric ulcer using a rat model. We examined macroscopic gastric injury, histological change of gastric mucosa, and ASIC3 expression in DRG neurons projecting to the stomach. We also assessed the effects of selective ASIC3 inhibitor APETx2 in the rat model.

2. Investigations and results

2.1. Value of intragastric pH in different groups

As shown in the Table, the value of intragastric pH was significantly lower in the WIRS group and the APETx2 treatment group than in the control group. Compared to the WIRS

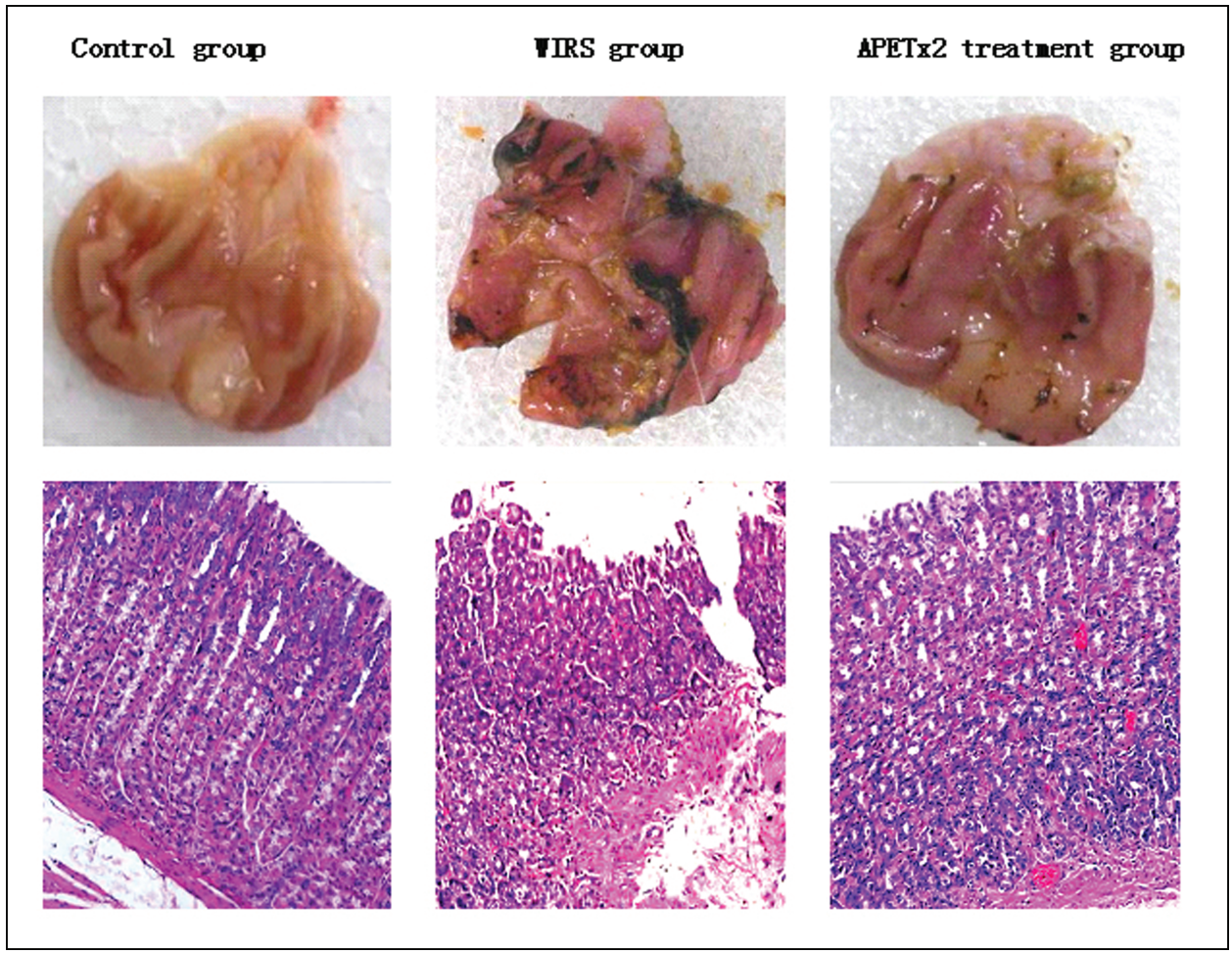

Fig. 1: Histopathology of the stomach sections. Six rats in each group were examined and representative images were shown. Upper: Gross appearance of gastric mucosa in three groups. Numerous hemorrhagic lesions were observed in the corpus mucosa and antral mucosa in WIRS group, and these lesions were attenuated in APETx2 treatment group. Lower: HE staining showed the damage to the surface epithelium and upper gastric pits involving exfoliation of surface mucosal cells into the gastric lumen and massive hemorrhagic necrosis in WIRS group. These lesions were attenuated in APETx2 treatment group (magnification × 200).

group, the intragastric pH value was significantly higher in the APETx2 treatment group. These data suggest that APETx2 could decrease gastric acidity.

2.2. Histopathological analysis of gastric injury in different groups

No inflammatory changes or gastric mucosal lesions were observed in the control group (Fig. 1). However, in the WIRS group we found serious gastric bleeding erosions, as indicated by mucosal hemorrhage, mucosal erosive lesion, and higher scores of ulcer index (Fig. 1, Table). Hemorrhage was observed mainly in the corpus mucosa and the antral mucosa, and the mucosa was disrupted. Massive hemorrhagic necrosis and the exfoliation of surface mucosal cells into gastric lumen were observed in the WIRS group. Notably, these features of mucosal lesions in WIRS group were significantly attenuated in the APETx2 treatment group, manifested by less mucosal hemorrhage, less inflammatory cell infiltration, and lower scores of ulcer index (Fig. 1, Table).

2.3. Expression of ASIC3 in DRG neurons in different groups

Next we performed immunohistochemical analyses of ASIC3 in DRG neurons among the three groups. In the control group, a weak staining of ASIC3 was detected in the membranes of some neurons. In the WIRS group, a strong staining of ASIC3 was observed in almost all neurons. Compared to the WIRS group, immunohistochemical staining of ASIC3 in DRG neurons was significantly attenuated in the APETx2 treatment group (Fig. 2). To confirm these results we performed quantitative real-time PCR. The results showed that ASIC3 mRNA levels were significantly lower in DRG tissues from the control group than in the other two groups. Compared to the WIRS group, ASIC3 mRNA level was significantly lower in DRG tissues from the APETx2 treatment group (Fig. 3A). Furthermore, Western blot analysis showed that ASIC3 protein level was low in DRG tissues of the control group, but was significantly increased in DRG tissues of the WIRS group. Compared to the WIRS group, ASIC3 protein levels were significantly lower in DRG tissues from the APETx2 treatment group (Fig. 3B).

3. Discussion

To our knowledge, for the first time we investigated ASIC3 expression in DRG neurons projecting to the stomach in rat model of WIRS, and assessed the *in vivo* efficacy of APETx2. Our results showed that compared to control rats, the rats exposed to WIRS exhibited lower intragastric pH values and higher UI scores. Histology analysis of gastric mucosa showed that the rats developed AGML lesions as described previously (Yasukawa et al. 2004). ASIC3 expression in DRG neurons projecting to the stomach was significantly up-regulated after exposure to WIRS. Administration of the ASIC3 inhibitor

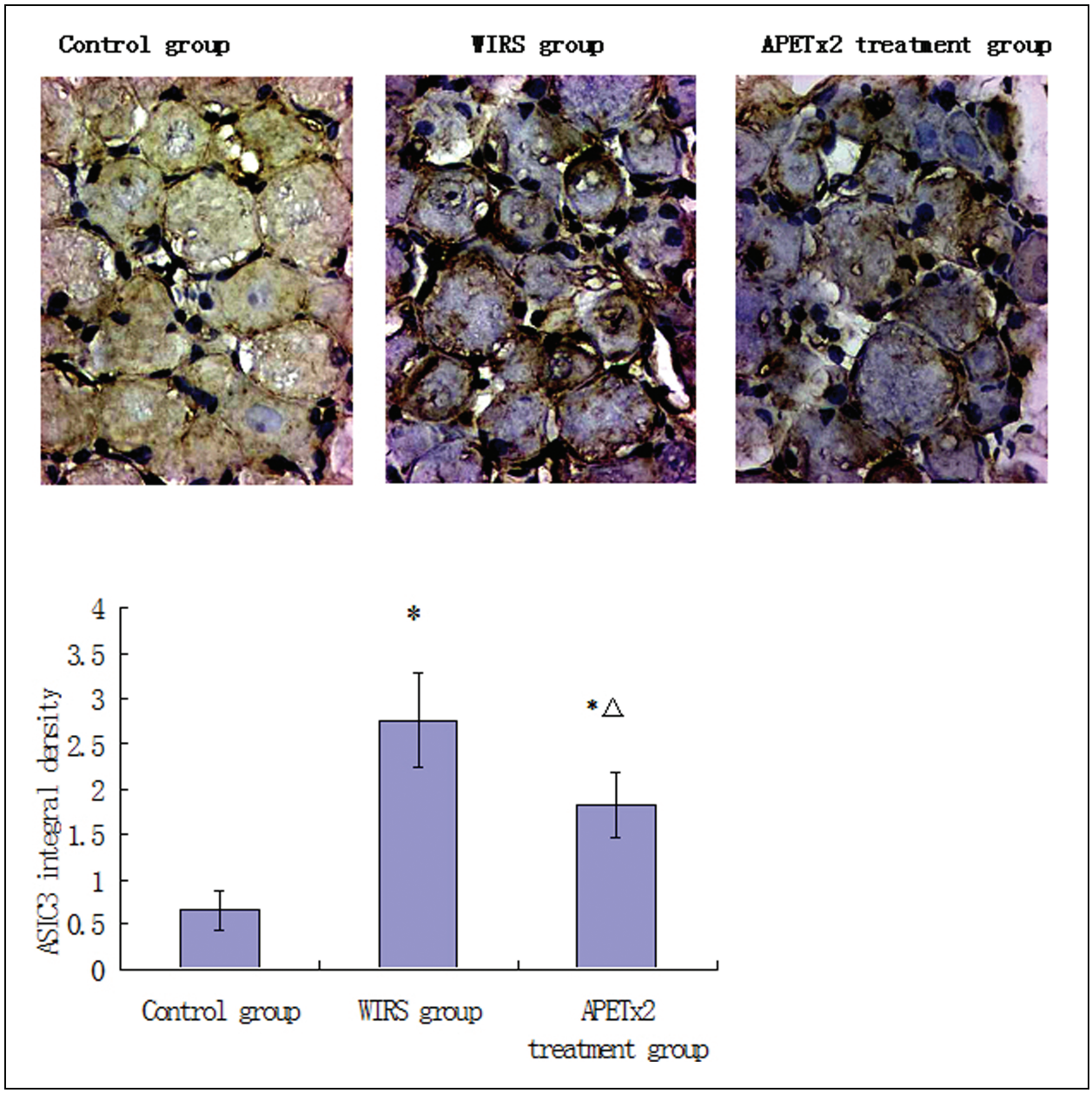

Fig. 2: Immunohistochemical staining of ASIC3 in DRGs sections. Six rats in each group were examined and representative images were shown on the upper. Strong staining of ASIC3 was indicated by dark brown. Original magnification: × 400. Lower: Semi-quantitative analysis of ASIC3 staining in three groups. Data were expressed as means ± SEM (n = 6). *$p < 0.05$ versus control group, $^{\Delta}p < 0.05$ versus WIRS group.

APETx2 resulted in a significant decrease in WIRS induced gastric mucosal lesions, gastric acidosis, and ulcer index in gastric mucosa, as well as reduced expression of ASIC3 in gastric afferents.

A variety of animal models of stress has been established and cold immobilization in the rat is a commonly used and clinically relevant model of stress induced ulceration (Silen 1988). Acidosis in the gastrointestinal tract is well-known to be caused by excess acid intake, excess gastric acid secretion, metabolic acidosis, and acidosis associated with other conditions such as ischemia and inflammation (Kemmerly and Kaunitz 2013). The disturbance of acid into the mucosa of the oesophagus, stomach or duodenum leads to the pain and the injury associated with gastro-oesophageal reflux and peptic ulcer disease (Huang and Hunt 1996). Our results showed that after WIRS, the intragastric pH dropped to 1.85.

ASICs regulate the capacity of afferent neurons to monitor acidosis (Holzer 2009; Waldmann et al. 1997). ASIC1b, ASIC2b and ASIC3 are expressed in distinct regions such as spinal and vagal afferent neurons innervating the gut and other visceral organs (Wemmie et al. 2006). ASIC3 plays a significant role in pain associated with tissue acidosis after injury or inflammation (Karczewski et al. 2010; Yen et al. 2009). Using a new model of gastric ulcer, our study provided new insights into the pathophysiology of stress ulceration associated with ASIC3 because increased AS1C3 expression at both mRNA and protein levels in thoracic DRGs after WIRS was detected by PCR, Western blot and immunohistochemical analysis.

To further confirm the contribution of ASIC3 to gastric acid hypersecretion and gastric mucosal lesion in the WIRS model, we employed APETx2, which could inhibit ASIC3 channels. We found that in the WIRS model, APETx2 treatment could significantly down-regulate ASIC3 expression in gastric afferents, decrease gastric acidity and prevent the occurrence of gastric mucosal lesions. Interestingly, a recent study reported that APETx2 could down-regulate the expression of ASIC3 in knee joint afferents (Izumi et al. 2012). However, the underlying mechanism by which APETx2 down-regulates the expression

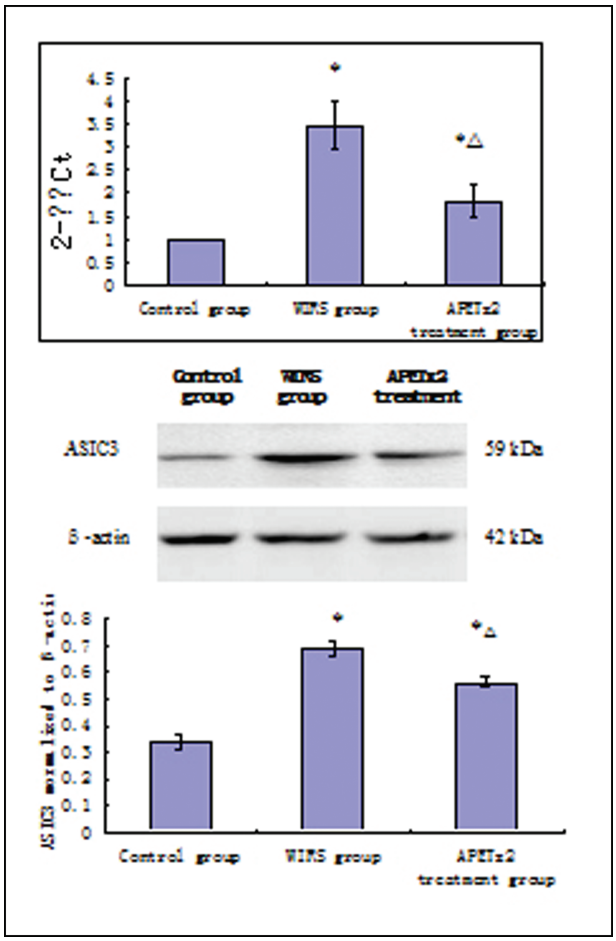

Fig. 3: Detection of ASIC3 expression level in DRGs tissues. Upper: Relative mRNA level of ASIC3 in DRGs tissues. Data were expressed as means ± SEM (n = 6). *$p < 0.05$ versus control group, $^{\Delta}p < 0.05$ versus WIRS group. Middle: Representative blots showing the expression of ASIC3 protein in DRGs tissues. β-actin was loading control. Lower: Densitometry analysis of ASIC3 protein level in DRGs tissues in three groups. Data were expressed as means ± SEM (n = 6). *$p < 0.05$ versus control group, $^{\Delta}p < 0.05$ versus WIRS group.

of ASIC3 is largely unknown. Further studies are needed to elucidate how a peptide inhibitor of ion channel inhibits the expression of ion channel.

Nevertheless, our study has several limitations. First, gastric histology and immunohistochemical analysis were only evaluated 6 h after APETx2 injection. Second, WIRS-induced AGML is a stress induced model. Other acid-induced gastric ulcer models may exhibit different characteristics of ulcer lesions (Schicho et al. 2004). Third, we only used one dose and one duration of APETx2 administration.

In conclusion, the present study is the first to characterize ASIC3 expression pattern in DRG neurons after WIRS. We found that high ASIC3 expression in gastric projected DRG neurons is strongly correlated with gastric mucosal lesion in the WIRS induced AGML model and that the ASIC3 inhibitor APETx2 decreases gastric acidity and alleviates the lesions. These findings suggest that ASIC3 inhibitors may be exploited as new agents for AGML treatment.

4. Experimental

4.1. Animals

The protocols were approved by the Ethics Committee of Guangzhou General Hospital of Guangzhou Military Command. All possible efforts were made to reduce both the suffering and number of animals used.

A total of 36 pathogen-free male Wistar rats (weight 170–220 g and 7–8 weeks old) were provided by the Animal Center of Guangzhou General Hospital and housed at 24 °C with a 12 h light/12 h dark cycle, and the rats had free access to water and regular chow. Before the experiments, each animal was housed in a single cage that had wire-net bottoms to avoid coprophagy. All animals were starved for 36 h before the experiments.

The rats were divided randomly into 3 groups (n = 12): (1) control group: rats were not exposed to any stress. (2) water immersion restraint stress (WIRS) group: rats were immersed up to the depth of the xiphoid process in a water bath at 18 °C for 6 h. (3) APETx2 treatment group: APETx2 (Alomone labs, RTA-100, Jerusalem, Israel) was dissolved in saline at 5 μg/ml. The rats received intraperitoneal (IP) injection of APETx2 (25 μg/kg body weight) at the time just before the WIRS procedure.

4.2. Tissue preparation

At the end of the experiments, the rats were anesthetized by IP injection of 10% chloral hydrate (400 mg/kg body weight). After the midline laparotomy was performed, the stomachs were secured by ligatures at the esophagus and duodenum. Then a cannula was placed in the left ventricle. Next, the right atrium was opened widely. Half of the rats (n = 6 for each group) were perfused with 250 ml cold heparinized 0.9% saline through the left ventricle. Next, both sides of thoracic DRGs (T8-T10) were obtained and immediately frozen in liquid N2 and later kept at -80 °C. The remaining rats underwent the same procedure as described above until the perfusion of 250 ml 0.9 % saline was performed. Then DRG tissue was fixed by 250 ml of cold 4% paraformaldehyde. After perfusion-fixation, both sides of thoracic DRGs (T8-T10) were removed and fixed in cold 4% paraformaldehyde overnight.

4.3. Intragastric pH measurement

The excised stomachs were opened along the greater curvature, and intragastric pH was measured with a pH meter (STARTER 3C Pro, OHAUS, USA).

4.4. Evaluation of gastric injury

The stomachs were fixed in 10% buffered formalin for 10 min, and the length and width of each hemorrhagic erosive lesion in gastric corpus mucosa were measured under a stereoscopic microscope (×10). Gastric damage was evaluated by ulcer index determination. Ulcer index was determined by a protocol-blinded investigator. For microscopic examination, the tissue was fixed in 10% formalin, dehydrated and imbedded in paraffin wax. The sections were cut as 5 μm thick and stained with hematoxylin and eosin.

4.5. Quantitative real-time PCR

Total RNA was isolated from DRG tissues using TRIZOL (Roche, Switzerland) following the manufacture's recommendations. The purity of total RNA was evaluated by 1% agarose gel electrophoresis. cDNA was made using Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). Quantitative real-time PCR was performed on Rotor Gene 6000 real-time PCR system (QIAGEN, Germany), using real-time SYBR Green PCR kit (Roche, Switzerland). The primers were as follows: ASIC3 sense 5'-TGAGAGCCACCAGCTTACC-3', and antisense 5'-GGCAGATACTCCTCCTGCT-3'; β-actin sense 5'-ATCATGTTTGAGACCTTCAACAC-3', and antisense 5'-TCTGCGCAAGTTAGGTTTTGTC-3'. PCR parameters were as follows: 45 cycles (15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C). β-Actin was used as internal control. The relative mRNA levels were represented as $\Delta Ct = C_{tgene} - Ct_{reference}$, and the fold change of gene expression was calculated by $2^{-\Delta\Delta Ct}$.

4.6. Western blot analysis

Thoracic DRG tissues were lysed in 20 mM Tris (pH 7.6) supplemented with 0.2% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulphonyl fluoride and 0.11 IU/ml aprotinin. The lysates were centrifuged at 12,000 × g for 30 min at 4 °C. Protein amount was measured by BAS method and aliquots of 20 μg of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with 5% skimmed milk at room temperature for 2 h, and incubated with rabbit polyclonal antibody against ASIC3 (ab77383; Abcam Biotech, 1:1,000 dilution) or β-actin overnight at 4 °C. After washing with tris-buffered saline with Tween 20 (TBST), the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG (1:1,000) for 1 h at room temperature, followed by washing with TBST. Then the membranes were exposed to X-ray film and mean pixel density was analyzed by Kodak Digital Science Image Analysis Software.

4.7. Immunohistochemical analysis

Paraffin sections of DRG tissues were dewaxed and incubated with 3% hydrogen peroxide for 10 min to block endogenous peroxide activity. Each section was incubated with normal goat serum at room temperature for 15 min and then washed with phosphate buffered solution (PBS). Next the sections were incubated with anti-ASIC3 antibody (ab77383; Abcam Biotech, 1:400 dilution) overnight at 4 °C and washed three times with PBS. Then the sections were incubated with horse anti-mouse IgG-HRP (1:400, as007, asbio) and diaminobenzidine, with counterstaining by hematoxylin. For negative controls, rabbit IgG. was used instead of primary antibody.

4.8. Statistical analyses

Data were analyzed by using SPSS software (Version 15.0, SPSS, Chicago, IL, USA). The comparisons of quantitative data among groups were performed using one-way analysis of variance followed by the Bonferroni post-hoc test. A value of $p < 0.05$ was considered significant.
Conflict of interest statement: There are no conflicts of interest.

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References

Duerksen DR (2003) Stress-related mucosal disease in critically ill patients. Best Pract Res Clin Gastroenterol 17: 327–344.

Holzer P (2009) Acid-sensitive ion channels and receptors. Handb Exp Pharmacol. 194: 283–332.

Holzer P, Wultsch T, Edelsbrunner M, Mitrovic M, Shahbazian A, Painsipp E, Bock E, Pabst MA (2007) Increase in gastric acid-induced afferent input to the brainstem in mice with gastritis. Neuroscience 145: 1108–1119.

Huang JQ, Hunt RH (1996) pH, healing rate and symptom relief in acid-related diseases. Yale J Biol Med 69: 159–174.

Izumi M, Ikeuchi M, Ji Q, Tani T (2012) Local ASIC3 modulates pain and disease progression in a rat model of osteoarthritis. J Biomed Sci 19: 77.

Jia YT, Wei W, Ma B, Xu Y, Liu WJ, Wang Y, Lv KY, Tang HT, Wei D, Xia ZF. (2007). Activation of p38 MAPK by reactive oxygen species is essential in a rat model of stress-induced gastric mucosal injury. J Immunol 179: 7808–7819.

Karczewski J, Spencer RH, Garsky VM, Liang A, Leitl MD, Cato MJ, Cook SP, Kane S, Urban MO (2010) Reversal of acid-induced and inflammatory pain by the selective ASIC3 inhibitor, APETx2. Br J Pharmacol 161: 950–960.

Kemmerly T, Kaunitz JD (2013) Gastroduodenal mucosal defense. Curr Opin Gastroenterol 29: 642–649.

Lingueglia E. (2007) Acid-sensing ion channels in sensory perception. J Biol Chem 282: 17325–17329.

Schicho R, Florian W, Liebmann I, Holzer P, Lippe IT (2004) Increased expression of TRPV1 receptor in dorsal root ganglia by acid insult of the rat gastric mucosa. Eur J Neurosci 19: 1811–1818.

Silen W (1988) Experimental models of gastric ulceration and injury. Am J Physiol 255: G395–402.

Sugiura T, Dang K, Lamb K, Bielefeldt K, Gebhart GF (2005) Acid-sensing properties in rat gastric sensory neurons from normal and ulcerated stomach. J Neurosci 25: 2617–2627.

Waldmann R, Champigny G, Bassilana F, Heurteaux C, Lazdunski M (1997) A proton-gated cation channel involved in acid-sensing. Nature 386: 173–177.

Wemmie JA, Price MP, Welsh MJ (2006) Acid-sensing ion channels: advances,questions and therapeutic opportunities. Trends Neurosc. 29: 578–586.

Yasukawa K, Kasazaki K, Hyodo F, Utsumi H (2004) Non-invasive analysis of reactive oxygen species generated in rats with water immersion restraint-induced gastric lesions using *in vivo* electron spin resonance spectroscopy. Free Radic Res 38: 147–155.

Yen YT, Tu PH, Chen CJ, Lin YW, Hsieh ST, Chen CC (2009) Role of acid-sensing ion channel 3 in sub-acute-phase inflammation. Mol Pain 5: 1.