TRPA1 and substance P mediate stress induced duodenal lesions in water immersion restraint stress rat model

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ABSTRACT

Background/Aims: Transient receptor potential ankyrin 1 (TRPA1) and substance P (SP), both expression in sensory neurons, have important roles in stress-induced duodenal lesions. The possible contribution of TRPA1 and SP to stress-induced duodenal lesions was explored by using the water immersion restraint stress (WIRS) rat model.

Materials and Methods: Western blotting, Real-time polymerase chain reaction (RT-PCR), and immunohistochemistry assay were used to evaluate the changes of TRPA1 and SP expression in the dorsal root ganglia (DRG, T8-11), the corresponding segment of the spinal cord (T8-11), and the duodenum in a duodenal lesions rat model. The SP concentrations of duodenal mucosa were investigated using an enzyme-linked immunosorbent assay (ELISA). Duodenal lesions were assessed according to histopathological changes. TRPA1 specific antagonist HC-030031 was intrathecally or intraperitoneally performed to suppress the expression of both TRPA1 and SP for evaluating the roles of TRPA1 and SP in duodenal lesions.

Results: In contrast to the control group, TRPA1 and substance P in the DRG (T8-11) and duodenum were up-regulated, and concentrations of SP in the duodenal mucosa were increased after WIRS (p<0.05), which are closely associated with duodenal lesions. SP concentrations in the duodenal mucosa were decreased and duodenal lesions were alleviated by pretreatment with TRPA1 antagonist HC-030031. We identified a protective role for HC-030031 in WIRS-induced duodenal lesions. Furthermore, we demonstrated that WIRS increased the concentrations of SP in the duodenal mucosa in a TRPA1-dependent manner. However, WIRS caused no significant changes of TRPA1 and SP in the spinal cord (T8-11) compared with the control group (p>0.05).

Conclusion: Our study indicates that TRPA1 antagonist HC-030031 alleviates duodenal lesions. TRPA1 is activated and sensitized, therefore concomitant neuropeptide SP is released, which exerts a critical role in inducing and maintaining duodenal lesions following WIRS in rats. This provides evidence that neuroimmune interactions may control duodenal injury. TRPA1 may be a potential drug target to inhibit the development of duodenal lesions by stress-induced in patients.

Keywords: Duodenal lesions, TRPA1, substance P, stress, inflammation, neuroimmune

INTRODUCTION

Duodenal lesions is a prevalent clinical problem. Although remedy for duodenal lesions is obtainable, duodenal lesions often relapses, and the number of patients suffered from this illness has maintained stability. Furthermore, there is a high risk that stress-induced duodenal lesions may develop into gastrointestinal (GI) bleeding, which is closely related to the increased morbidity and mortality in critical patients (1). Therefore, there is a need for more beneficial and preventive therapies for duodenal lesions. In this study, an animal model of duodenal lesions induced by the water immersion restraint stress (WIRS) is used to better understand the pathogenesis of duodenal ulcer or even bleeding in humans subjected to excessive stress.

Although several studies have reported that transient receptor potential vanilloid 1 (TRPV1) expressing in primary sensory neurons is critical in the induction and diffusion of abnormal immune responses in colitis (2), the pathogenesis of stress-induced duodenal lesions is not well understood. Many studies have revealed that substance P (SP) abundantly exists in the central and peripheral nervous system. Hence, SP is related to various physiological and pathophysiological processes including inflammation, stress regulation and immune regulation (3,4). SP is also richly present in the GI tract. The most of SP in a circular system evidently stems from the intestine (5). Furthermore, quantitative analyses showed that the highest concentrations of SP in the gut are found in the duodenum and jejunum (6). SP in the GI tract expresses both in intrinsic enteric
neurons and in extrinsic nerves, which belong to the primary sensory neuron. And therefore, SP exerts a major role in regulating the intestinal motility and blood flow (7). SP release from sensory nerve terminals can produce neurogenic inflammation including vasodilatation, edema, plasma protein extravasation, and neutrophil accumulation (8,9).

Transient receptor potential ankyrin 1 (TRPA1) was initially characterized as a thermoreceptor activated by temperatures ≤17°C (10). Therefore, TRPA1 was originally proposed as a pain receptor for cold (11). Tissue distribution of TRPA1 mRNA revealed abundant expression in the GI, from the stomach to the colon, including enteric nerves, and dorsal root ganglia (DRG) in humans, mice, and rats. TRPA1-specific staining in situ hybridization was observed in the rat duodenal epithelial layer. The numbers of TRPA1 staining cells in the duodenum was approximately 70% in rats (12). Hence, TRPA1 is vital in visceral mechanical sensation and inflammatory hyperalgiesia (11,13). In addition, TRPA1 and SP were abundantly co-expressed in thoracolumbar DRG neurons and expressed in duodenum (6,14). These data indicated that TRPA1 activation in primary afferent neurons might induce SP release, which can elicit neurogenic inflammation, as well as promote duodenal lesions. To sum up, the roles of TRPA1 and substance P in stress-mediated duodenal lesions have aroused our great interest.

Hereby, we stress on the fact that TRPA1 is a critical sensor of stress and tissue injury as well as a multi-purpose sensor of deleterious signals. Furthermore, TRPA1 can be activated by oxidative stress metabolites and endogenous inflammatory mediators (15). Thus, we hypothesized that WIRS could induce the activation of TRPA1 in sensory neurons and duodenum, followed by the subsequent release of SP, which is closely related to inducing and maintaining duodenal lesions.

**MATERIALS AND METHODS**

**Animals**
The ethics protocols were approved by the Ethics Committee of Guangzhou General Hospital of Guangzhou Military Command (Decision Date: 05.24.2016/Decision No: 20160524-01). Male Wistar rats (180-220 g, 6-8 weeks old) were obtained from the Animal Experimental Center of Guangzhou General Hospital and housed in 24°C with a 12 h light/12 h dark cycle. Food (except for water) was not provided for 24 h before experiments. All possible efforts were made to decrease both the suffering and number of animals used.

**Induction of duodenal lesions and HC-030031 treatment**

Water immersion restraint stress is commonly used as an experimental model of emotional stress-mediated acute gastric mucosal lesions based upon its clinical relevance and reproducibility. A WIRS-induced duodenal injury model was shaped using the method of Murakami S (16) and Takagi (17). Rats were immobilized in a captive cage and then immersed up to their xiphoid in a water bath at 16°C±1. After 6 h, rats were immediately anaesthetized with 10% chloral hydrate; the DRG, duodenums, duodenal mucosa and spinal cords were excised.

The rats were divided into six groups. Group 1 (Control group) did not experience any procedures. Group 2 (WIRS group) underwent WIRS procedures. In group 3 (ITIH group) and group 5 (ITIH-Control group), a small incision was made at the back of the rat’s lumbar 4/5 stage, and PE-10 catheter at the level of T9-10 DRG was put into the subarachnoid space according to described previously (15). Rats showing motor or neurological dysfunction following catheter insertion were sacrificed. In group 3 (ITIH group), the 10 µL TRPA1 inhibitor HC-030031 (Sigma, H4415, USA, HC-030031 dissolved in pure dimethyl sulfoxide at 10 µg/µL) was intrathecally injected, and then flushed with 10 µL saline 30 min before WIRS (15). In group 4(IPIH group), HC-030031 with 150 mg/kg (HC-030031 dissolved in 0.5%-methyl cellulose) was intraperitoneally injected 1h before WIRS (15). In group 5 (ITIH-Control group) and group 6 (IPIH-Control group), dimethyl sulfoxide and 0.5%-methyl cellulose were used as negative control.

**Western blotting (WB)**
The excised DRG and spinal cords were homogenized in radio immunoprecipitation assay Lysis Buffer. Polyacrylamide gel electrophoresis separated proteins. Western blotting was administered with the primary antibodies: TRPA1 (1:1000, Alomone labs, ACC-037, Jerusalem, Israel), and GAPDH (1:5000, Abcam, ab9485, Cambridge). Immunoreactive signals were detected using enhanced chemiluminescence Plus WB substrate. Protein quantification was normalized to GAPDH.

**Real-time polymerase chain reaction (RT-PCR)**
Total RNA was extracted from the excised DRG (T8-T11) with TRIZOL (Sigma, T9424, USA), and was reverse transcribed into cDNA using the Super Script III First-Strand Synthesis System (Invitrogen,18080051, Carlsbad, CA) and oligo (dT) primers. RT-PCR was performed on a LightCycler®480 real-time PCR system 1.5 (Roche...
diagnostics, Neuilly sur Seine, France), using Ex Taq (TAKARA, DRR081A, Otsu, Shiga, Japan). The primers were as follows: rat SP primer (forward: 5’-TTCTCTTCCCTT-GCTGC-3’, reverse: 3’-GTCTGAGGAGGTCA- CATT-5’). β-actin (forward: 5’-GATCAAGATCTCCCTGC-3’, reverse: 3’-AGGGTGTAAAACGCTCA-5’). β-actin was used as an internal control. The mRNA levels of SP gene was calculated after normalizing to β-actin by the comparative CT method (2-ΔΔCT).

**Enzyme-linked immunosorbent assay (ELISA)**

The concentrations of SP in the duodenal mucosa was determined by using the SP ELISA Kit (R&D SYSTEMS, KGE007, USA). Duodenal mucosa was homogenized in phosphate buffer saline. Next, 50 µL the supernatant after centrifugation of duodenal mucosa was added into each well. The concentrations of SP in duodenal mucosa was normalized by the quantity of proteins.

**Histological examination (HE)**

Following anaesthesia, rats were fixed with 250 mL saline and 300 mL of 4% paraformaldehyde (PFA) solution from the left ventricle. DRG (T8-T11), and spinal cords (T8-T11) were removed and fixed in 4% PFA overnight, then embedded in paraffin. 3µm slices of duodenum were stained with HE for histopathological analysis. The images of HE staining were obtained using a digital microscope (Olympus, BX51, Japan) and the histopathological scores of the duodenal lesions were determined as previously described (18,19). The severity of duodenal lesions following WIRS was scored from 0 to 5 as follows: 0- normal mucosa villi; 1- capillary congestion; 2- moderate lifting of duodenal epithelial layer; 3- massive duodenal epithelial lifting; 4- denuded villi & dilated capillaries; 5- disintegration and digestion of lamina propria; or hemorrhage, and ulceration.

**Immunohistochemistry (IHC)**

For IHC, 3µm sections of duodenum, DRG, and spinal cord were dewaxed and hydrated. Sections were incubated for 12 hours at 4°C with rabbit anti-TRPA1 (1:400, Alomone labs, ACC-037, Jerusalem, Israel) or mouse anti-SP (1:200, R&D SYSTEMS, MAB4375, USA). Sections were incubated with secondary antibody K5007 (DAKO, Denmark). Sections were imaged with a digital microscope (Olympus, BX51, Japan). At last, immunolocalization of TRPA1 and SP receptors was evaluated by using Image-Pro Plus 6.0 software (version 6.0, USA).

**Statistical analysis**

All data are expressed as the mean±SEM. All statistical analyses were performed using Statistical Program for Social Sciences version 21.0 (IBM Corp.; Armonk, NY, USA). The comparisons of quantitative data among groups were performed using either Independent-Samples t-test or one-way ANOVA followed by Dunnett’s T3 test. p<0.05 were considered to be significant. All data were processed in GraphPad Prism 5 software (La Jolla, USA).

**RESULTS**

**HC-030031 alleviated WIRS-induced duodenal lesions**

Using hematoxylin and eosin stain histopathology, we determined duodenal lesions histological scores (Figure 1.g.). Intact duodenum (control) did not display microscopic lesions. Stress exposure induced the formation of duodenal lesions in other five groups. The severe damage of the duodenum induced by WIRS was observed using histological examination (Figure 1). In WIRS group, numerous duodenal lesions were apparent as follow: acute erosive hemorrhagic lesions, as well as massive hemorrhagic necrosis, marked capillary congestion, denuded villi and dilated capillaries, massive duodenal epithelial lifting, and tissue looseness, edema, local abscession of laminae propria with inflammatory cell infiltration, and duodenal glands structural disorder, gland atrophy and reduction and local deficiency. Occasionally accompanied by cell degeneration, cell edema, pale cytoplasm in muscular layer. These lesions were attenuated in HC-030031 treatment group (ITIH group and IPIH group, p<0.001, Figure 1.c, d.). However, vehicle control (Figure 1.e, f.) did not alleviate WIRS-induced duodenal lesions, compared with the WIRS group (p>0.05).

At the macroscopic level, WIRS caused extensive visible hemorrhage and reddening on the duodenal luminal surface (Figure 1.i.). Taken together, these results suggest that WIRS induced acute lesions, inflammatory response, and structural changes in the duodenum. Importantly, the pretreatment of duodenal lesions with HC-030031 effectively attenuated the severity of damage in the duodenum by WIRS induction (Figure 1. c, d.).

**WIRS induced up-regulation of TRPA1 and SP expression in DRG and duodenum**

Water immersion restraint stress up-regulated TRPA1 in DRG (T8-11) and duodenum (Figure 2), and SP in DRG
(T8-11) and duodenum (Figure 3), compared with the control group (p<0.001). However, western blotting and IHC revealed no significant differences in TRPA1 and SP in spinal cords (T8-11) between WIRS and control groups (p>0.05, Figure 4).

**HC-030031 reduced the SP concentrations in duodenal mucosa**

Substance P concentrations in duodenal mucosa were measured by using ELISA. The WIRS significantly increased SP concentrations in the duodenal mucosa, compared with the control group (p<0.001, Figure 5. a, b). And administration of HC-030031 (Figure 5. c, d) significantly reduced SP concentrations in the duodenal mucosa, compared with the WIRS rats (p<0.001). However, vehicle control (Figure 5. e, f) did not reduce SP concentrations in the duodenal mucosa, compared with the WIRS group (p>0.05).

**DISCUSSION**

In the present study, we found that WIRS caused the duodenal lesions and up-regulated TRPA1 and SP in DRG.
neurons and duodenum, as well as increased SP concentrations in duodenal mucosa in rats. Duodenal lesions was closely related to increased expression of TRPA1 and substance P in DRG neurons and duodenum, and SP concentrations in duodenal mucosa. The duodenal lesions by WIRS-induced were effectively attenuated and SP release was almost entirely blocked in all HC-030031 pretreated groups (ITIH group and IPIH group). Therefore, we identified a protective role for HC-030031 in WIRS induction duodenal lesions. Furthermore, we demonstrated that WIRS mediated SP release in the duodenal mucosa, in a TRPA1-dependent manner. These are consistent with Schwartz ES et al. (20) and Engel MA et al. (21) reported results. Here, we suggest that TRPA1 sensitization and activation, and concomitant neuropeptide SP release play a prominent part in the induction and persistence of inflammation in the duodenum by WIRS.
duodenal lesions. These finds suggest that WIRS induced activation of TRPA1 is the first step in a cascade of events that result in duodenal damage.

We also identified a TRPA1 and SP dependent sensory neuronal mechanism in WIRS-induced duodenal lesions. GI secretion and motility are regulated not only by neurotransmitters and hormones, but also by neurons (12). In many instances chronic, sustained, unpredictable stress, whether physical or psychological, is the most deleterious, contributing to immune and endocrine dysfunction (22). The neuroendocrine hormones released during a stressful event could cause alterations in immune function and immune imbalance (23). It’s long been known that the nervous and the immune system can interact through bidirectional communication. SP is considered a major mediator of neurogenic inflammation and links the nervous and the immune system. TRPA1 presents on afferent nerves, which can detect and react to potentially damaging stimuli, such as stress, physical, and chemical stimuli. These neurons-stimulated send signals and concomitantly release neuropeptides, such as SP, promoting neurogenic inflammation and the enhancement of mast cell activation by neuron-induced. Mast cells (MC) are located in close proximity to SP containing sensory nerves (24,25). The GI tract is innervated by extrinsic and intrinsic enteric neurons, which both contain SP. Cell bodies of extrinsic enteric neurons are found in the DRG and their terminals innervate mainly the arterial vascular system. In this way, SP released from sensory neurons can directly activate MC, which can release various pro-inflammatory substances (such as histamine, 5-HT and leukotrienes), which lead to the neutrophil infiltration, leukocyte-platelet accumulation, vasodilation, and the boost of vascular permeability and vascular protein leakage (26). In addition, SP induces neutrophil infiltration and participates in inflammatory response through degranulation of MC. Previous reports have demonstrated that SP release contributes to various inflammatory diseases via different effects on immune cells (4,27).

The cell bodies of splanchnic nerves are located in the thoracolumbar DRG. These sensory afferent nerves can send signals to the central nervous system, which is the main pathway. Furthermore, TRPA1 channels functionally expressed in visceral afferent sensory neurons and intestinal epithelial cells that could influence the intensity of the duodenal lesions. The unmyelinated C afferent nerve fibers may be the most important sub-type in MC-activated sensory afferent in the GI tract (26). TRPA1 and SP are abundantly expressed by these C afferent nerve fibers and extensively co-expressed in DRG, which may play an important role in regulating GI motility, and the innate immune system, respectively. TRPA1-activated on primary sensory neurons leads to afferent and efferent signals. Then, an influx of [Na+]i and [Ca2+]i through TRPA1 causes an action potential, and a local neuropeptide SP release in sensory. The GI tract SP immunoreactive nerve networks are found around blood vessels. So these neuropeptides in these blood vessels trigger the inflammatory response, vasodilation, and vascular leakage (28,29). It is well established that the release of SP from afferent terminals conduces to neurogenic inflammation, as well as hyperalgesia in rodents, by causing capillary vasodilation, plasma extravasation, leukocyte infiltration in intestine, and partly with sensitizing co-expressed TRPA1. Meanwhile, nerve fibers, most especially SP expression in sensory neurons, become physically closer and apposed to MC. GI mucosal MC also express TRPA1 (29). Then, stress-induced TRPA1 activation and concomitant neuropeptides SP released from afferent nerve endings which can directly stimulate MC, and cause MC mediator release, as well as alter function by piecemeal- or classic degranulation (24). It’s of interest that organs containing a high concentration of SP, such as the intestines are thought to be more susceptible to dysfunction from inflammation (30). Furthermore, the previous studies showed that SP induced neutrophil infiltration in rat mesenteric postcapillary venular endothelium by MC-activated (24).
TRPA1 and SP are expressed in intrinsic and extrinsic primary afferent neurons; these are critical in visceral pain and stress response (31,32). Here TRPA1 detects diverse noxious compounds which elicit pain and neurogenic inflammation (33). In addition, TRPA1 expression has been described in rodent and human small intestine where it is most abundant compared to other organs in rat (12). Herein, WIRS specifically activates TRPA1 on sensory neurons and causes SP release in the DRG and duodenum. This then leads to a persistent immune response and results in duodenal lesions. HC-030031 ameliorates duodenal lesions through its inhibition of TRPA1 activation and proinflammatory TRPA1-mediated SP release, resulting in amelioration of neurogenic inflammatory effects. However, there were no significant differences in TRPA1 and SP expression in spinal cords (T8-11) between WIRS and control groups. This may be because both TRPA1 and SP are expressed and co-expressed in DRG neurons (14). However, in the spinal cord (T8-11), TRPA1 is notably expressed in grey matter glial cells, whereas SP is not expressed in glial cells of the spinal cord and is richly expressed in grey matter neuron.

TRPA1 and SP co-express in the extrinsic primary afferents (34). SP may reach the duodenum from the spinal dorsal root ganglia joining the splanchnic nerves and visceral blood vessels as well as through vagal nerves. Various GI reactions can be affected by vagal afferents and enteric nerves which are stimulated by SP. Vagal nerves stimulation is reported to attenuate the production of proinflammatory cytokines and inhibit the inflammatory processes in various experimental models (35). Abundant TRPA1 expression was detected in vagal afferent fibers (36). Thus, TRPA1-mediated effects on duodenal lesions could be vagal nerves dependent. Duodenal lesions was attenuated when TRPA1 was pharmacologically inhibited by intrathecal and intraperitoneal administration of HC-030031. This may have lead to desensitization of TRPA1 in sensory nerve fibers, thus explaining protection from duodenal lesions. Furthermore, the protective responses triggered by sensory neurons in the digestive system include alterations in gastrointestinal blood flow, secretion and motility as well as regulating immune function (37). The role of sensory neurons in recovery and healing of gastro-duodenal ulcers was showed. Recovery of gastric lesions induced by WIRS, indomethacin, ischaemia/reperfusion, or concentrated ethanol was postponed in animals with functional deletion of sensory nerves. The delay in ulcer healing was found to be associated with a change of both growth factors and inflammatory mediators (38). SP is known to stimulate the production of IL-8 and IL-6 in human colonic epithelial cells (39). Stress firstly causes gastrointestinal vasoconstriction, subsequently followed by vasodilation, which is similar to ischemia-reperfusion and hypoxia-reoxygenation. The intestinal injury is found to be caused by multiple factors, comprising oxygen free radical formation (OFRF), inflammatory cytokine release, reactive oxygen species, and neutrophil infiltration in damaged tissues and cells (18). It triggers a series of events that may result in increasing intestinal epithelial permeability and destructing the mucosal barrier function.

The activation of TRPA1 is a double-edged sword regarding its role in intestinal inflammation. In models of experimental colitis, it was found that activation of TRPA1 and consecutive neuropeptide release induced colitis (21). However, sustained repetitive activation finally resulted in inhibition of inflammation as well as nociceptive responses through desensitization of nociceptive peptidergic sensory neurons (40). In healthy and inflamed colons of human and murine, TRPA1 and also TRPV1 were located in epithelial cell and macrophages (41). It is possible for TRPA1 in macrophages to mediate the anti-colitogenic function, because macrophages are main makers of tumour necrosis factor (TNF)-α and TRPA1-activated was able to down-regulate TNF-α in the distal colon during colitis. A recent study emphasized that the effects of extra-neuronal TRPV1 and TRPA1expression played a key role in the etiopathogenesis of intestinal inflammation and led to a complicated cross-linking reaction of different cellular compartments in this context (42). Furthermore, preparing different models of mouse colitis including both genetic deletion of interleukin (IL)-10 mice and adoptive T-cell-transferred models of colitis, it was found that the TRPA1 knockout in CD4+ T cells resulted in intestinal inflammation through the transcription factor T-bet-induced, which subsequently could raise products of the interferon (IFN)-γ and IL-2 and cause a higher ability to discriminate into T helper 1 effector cells (43).

In summary, we found that TRPA1 and SP play an important role in the physiology and pathophysiology of duodenal lesions. We demonstrated that duodenal lesions was ameliorated and SP release was inhibited in WIRS rats injected with HC-030031. Therefore, TRPA1 may be a potential drug target to inhibit duodenal lesions by stress-induced in patients. These findings may contribute to the discovery of drugs for duodenal lesions treatment. However, the TRPA1 block did not completely inhibit duodenal lesions, which suggests other SP release mechanisms besides TRPA1 activation contribute to duodenal lesions. Further research is necessary to con-
firm the effect of TRPA1 agonist and SP antagonist on duodenal injury. Although we concluded that substance P was critical in WIRS induced duodenal lesions, the therapeutic potential for stress-induced duodenal lesions was not brought up.

**Ethics Committee Approval:** Ethics committee approval was received for this study from the Ethics Committee of Guangzhou General Hospital of Guangzhou Military Command (Decision Date: 05.24.2016; Decision No: 20160524-01).

**Informed Consent:** N/A.

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