Desensitization of Capsaicin-Sensitive Sensory Neurons in Rat Stomachs on Chronic Treatment with Sodium Taurocholate

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ABSTRACT—We examined the effects of chronic treatment with 10 mM sodium taurocholate (TC) on gastric functions, capsaicin-sensitive afferent neurons and the gastric mucosa in male rats. Stomachs were mounted in Lucite chambers, and then the transmucosal potential difference (PD), luminal pH and gastric mucosal blood flow (GMBF) in response to TC or capsaicin was determined. In normal animals, 10 mM TC caused a reduction in PD, and increases in luminal pH and GMBF. Capsaicin (1 mg/ml) produced an apparent increase in GMBF without any change in PD or luminal pH. After 4- or 12-week treatment with TC, the basal PD was significantly reduced, and the luminal pH tended to increase. The increase in GMBF in response to TC or capsaicin was profoundly suppressed in TC-pretreated animals. The calcitonin gene-related peptide release in response to capsaicin was significantly reduced after 4 weeks treatment with TC. There were no microscopical changes in the oxyntic mucosa until 4 weeks after TC treatment except for exfoliation of surface cells. However, an increase in inflammatory cell infiltration was observed 12 weeks later. We conclude that chronic treatment with TC causes desensitization of capsaicin-sensitive afferent neurons and reduces GMBF, which may result in the production of gastritis.

Keywords: Gastritis, Sodium taurocholate, Capsaicin-sensitive afferent neuron, Calcitonin gene-related peptide, Gastric mucosal blood flow

Chronic treatment with 5 mM sodium taurocholate (TC) for >3 months induces gastritis in rats (1, 2). We also observed that treatment of rats having undergone unilateral vagotomy with 5 mM TC for 6 months induced gastritis that involved decreases in the number and density of parietal cells and an increase in inflammatory cell infiltration (3). The mechanism by which chronic treatment with TC results in such gastritis remains unclear. There is increasing evidence that capsaicin-sensitive afferent neurons (capsaicin-sensitive neurons) participate in the protective mechanism against acute gastritis or gastric ulceration (4-7), probably through the release of the calcitonin gene-related peptide (CGRP) (8-10). Therefore, it is possible that acute or chronic depletion of CGRP from the gastric or duodenal mucosa may weaken the mucosal integrity, leading to the production of gastritis and ulcers. Indeed, ulcerogens such as acetic acid, concentrated ethanol and cysteamine significantly depleted CGRP from the peripheral endings of capsaicin-sensitive neurons, suggesting a causal relationship (11-13). It was of interest to examine whether or not chronic treatment with TC has any damaging effect on capsaicin-sensitive neurons which leads to disturbance of gastric functions, thereby eventually resulting in the production of gastritis. In this study, we determined the changes in gastric functions, the amount of CGRP released from the gastric mucosa and gastric mucosal histology in TC-treated animals. An abstract of part of this study has already been published (14).

MATERIALS AND METHODS

Animals

Male Donryu rats (Japan SLC, Shizuoka) weighing 230–280 g were used in all experiments. The animals were kept in individual cages with raised mesh bottoms to prevent coprophagy, and they were deprived of food, but allowed free access to tap water for 18 hr before the experiments.

TC treatment and functional ablation of capsaicin-sensitive neurons

Normal animals or animals pretreated with TC for certain periods were used to determine the local effect of TC.
or capsaicin on the exposed gastric mucosa. In the case of TC treatment, the animals were allowed free access to a bottled supply of water containing 10 mM TC or water alone for 1, 2, 4 or 12 weeks. Other investigators (1, 2) and our group (3) used 5 mM TC in previous studies, but the concentration of TC was increased to 10 mM in this study to ensure the production of the possible dysfunction of capsaicin neurons within a short period. In some experiments, ablation of capsaicin-sensitive neurons was performed by s.c. injection of capsaicin once daily for 3 consecutive days (20, 30 and 50 mg/kg) 2 weeks before the experiments (capsaicin pretreatment) (15). Capsaicin injections were given under ether anesthesia, and the rats were pretreated with terbutaline (0.1 mg/kg, i.m.) and aminophylline (10 mg/kg, i.m.) to counteract the respiratory impairment associated with capsaicin injections. To check the effectiveness of the treatment, a drop of a 0.1 mg/ml solution of capsaicin was instilled into one eye of each rat and then the protective wiping movements were counted, as previously reported (16). Control animals were also treated with terbutaline and aminophylline, but received the vehicle alone instead of capsaicin.

**Determination of gastric functions**

Intact rats were anesthetized with urethane (1.25 g/kg, i.p.), and then their stomachs were exposed through a midline incision and mounted in Lucite chambers according to the previously published method (17). Under these conditions, the mucosa was perfused at the flow rate of 1 ml/min with saline at 37°C; the transmucosal potential difference (PD) was determined using two agar bridges, one positioned in the chamber and the other in the abdominal cavity; and the pH of the gastric effluent (luminal pH) was determined with a pH glass electrode of the flow type (Model 6901-25T; Horiba, Kyoto). Changes in PD were continuously monitored with a recorder (Model U-228; Nippon Densi Kagaku, Tokyo) simultaneously with the pH (Model 6901-25T; Horiba, Kyoto). The rats were killed and their stomachs were removed. The forestomach and antrum were dissected out, and the oxyntic mucosa was inverted (mucosal side out). These tissues were incubated in test tubes containing 2 ml of a modified Krebs-Henseleit solution gassed with 5% CO2 in O2 maintained at 37°C. The solution contained the following: 110 mM NaCl, 4.7 mM KCl, 1.0 mM CaCl2, 1.15 mM MgCl2, 25 mM NaHCO3, 5.6 mM glucose and 10 mM Hepes. Capsaicin (10-5 M) dissolved in 10% ethanol (0.1 ml) or vehicle alone was added to the incubated tissue. One hour later, the incubated solution was centrifuged at 3,000 rpm for 5 min at 4°C. The CGRP content of the supernatant was determined by chemiluminescence enzyme immunoassaying. The procedure was as follows: purified goat anti-rabbit IgG was fixed onto the wells of microtiter plates, followed by blocking with Blockace (Yukijirushi Nyugyou, Tokyo). After washing with 50 mM Tris-HCl (pH 7.5), a mixture of the supernatant and biotinylated CGRP (b-CGRP) was added to the wells, followed by standing at 4°C overnight. Excess b-CGRP was washed out. Alkaline phosphatase-conjugated streptavidin was added to the wells at 30°C for 1 hr, and then the wells were washed with 50 mM Tris-HCl (pH 7.5) 5 times. The reaction for detection was initiated by the addition of the AMPPD solution. After 16 min at room temperature, the luminescence of AMPPD was measured with a luminometer (LB9501; Berthold, Wiesbaden, Germany).

**Histological studies**

The rats were killed and their stomachs were removed under ether anesthesia. The stomachs were then opened along the greater curvature and pinned on a cork board. Tissue samples were obtained from 4 parts of the oxyntic mucosa, and they were immersed in neutral 10% formalin for 2 days. They were then embedded in paraffin and sectioned (4 μm) by the routine method. Each section was stained with hematoxylin and eosin. The mucosal thickness (μm) was measured at 100 power magnification, and the number of parietal cells was counted per unit area (2.5 mm square) at 400 power magnification using an
ocular eye grid. The density of parietal cells was calculated as the number of parietal cells/total cell number per unit area (2.5 mm square). The degree of inflammatory cell infiltration and the extent of fibrosis were evaluated, grades 0, +1 or +2, at 100 power magnification. Determination of all parameters was performed by a person who was unaware of the treatment.

**Drugs**

Urethane (Tokyo Kasei, Tokyo), sodium taurocholate (Difco, Detroit, MI, USA), capsaicin (Wako, Osaka), aminophylline (Neophylline; Eisai, Tokyo) and terbutaline (Bricanyl; Fujisawa, Osaka) were obtained commercially. Capsaicin was dissolved in a Tween 80-ethanol solution (10% ethanol, 10% Tween 80 and 80% saline) for s.c. injection and suspended in a 0.5% carboxymethylcellulose solution (CMC; Nacalai Tesque, Kyoto) for mucosal application. Each drug was prepared immediately before use, and it was given in a volume of 0.5 ml/100 g of body wt. in the case of i.p., i.m. and s.c. administration. Control animals received the vehicle alone.

**Statistics**

The data are presented as the means±S.E. for 6 rats per group. Statistical analyses were performed using the two-tailed Dunnett’s multiple comparison test (19), values of P<0.05 being regarded as significant.

**RESULTS**

**Effect of TC or capsaicin in the stomachs of normal animals**

The gastric mucosa mounted in a Lucite chamber generated a PD of about −35 to −38 mV, secreted acid to maintain the luminal pH at about 3.0−3.5, and maintained a GMBF of about 5−15 ml/min/100 g. These values remained relatively constant for 2 hr and did not significantly change after application of the vehicle solution. Under these conditions, exposure of the mucosa to 10 mM TC caused a reduction in PD of 41% and an increase in luminal pH of 27% (Fig. 1). On the other hand, there was a significant increase in GMBF of 49% at the end of the application of 10 mM TC compared to the control value (Fig. 1). On the other hand, there was a significant increase in GMBF of 49% at the end of the application of 10 mM TC compared to the control value (Fig. 2). Local application of capsaicin (1 mg/ml) to the mucosa caused no appreciable change in either PD or luminal pH (data not shown), but significantly increased GMBF by about 80% during the application (Fig. 2). Accordingly, we determined the effect of capsaicin on GMBF alone in rats pretreated with TC in the following experiment.

**Effect of TC or capsaicin in the stomachs of capsaicin pretreated rats**

The functional ablation of capsaicin-sensitive neurons with a large amount of capsaicin did not cause any change in PD, luminal pH or GMBF under the basal conditions. However, capsaicin pretreatment remarkably attenuated the increase in GMBF induced by both TC and capsaicin (Fig. 2). The maximal increases in GMBF in response to TC and capsaicin were 13% and 19%, respectively. These changes were not significantly different from the basal values observed before the exposure. The decrease in PD and the increase in luminal pH on TC application in capsaicin pretreated rats did not change, but the recoveries of both the PD and pH values were significantly delayed as compared with those in normal rats (Fig. 1).
Effect of TC or capsaicin in the stomachs of TC-treated rats

Animals well tolerated the TC treatment for up to 12 weeks, the weight gain being the same as that in the control group. In addition, there was no gross damage to the oxyntic mucosa throughout the experimental period. One week treatment with TC caused no change in PD, luminal pH or GMBF in the basal state (Fig. 3 and 4). When TC was locally applied, it significantly caused a reduction in PD and increases in luminal pH and GMBF as compared with the basal values. On 2 weeks treatment, the basal PD was slightly reduced, without the basal luminal pH and GMBF being affected. Local application of TC induced a reduction in PD and increases in luminal pH and GMBF as compared with the basal values. On 2 weeks treatment, the basal PD was slightly reduced, without the basal luminal pH and GMBF being affected. Local application of TC induced a reduction in PD and increases in luminal pH and GMBF as compared with the basal values. After 4 weeks treatment, the basal PD was significantly reduced from -36 mV to -26 mV, but the luminal pH tended to increase from 3.4 to 3.9. Immediately after TC application, the PD and luminal pH values were not significantly different from those observed with 1 or 2 weeks treatment, although the degrees of the changes were smaller. However, the increase in GMBF was apparently reduced from 57.3% in the controls to 20.6% in TC-treated rats. After 12 weeks treatment, the changes in PD and luminal pH in the basal and TC-applied states were similar to those observed with 4 weeks treatment. It is noteworthy that GMBF did not increase after TC application.

The local application of capsaicin to the gastric mucosa of rats pretreated with TC for 1 or 2 weeks caused a marked increase in GMBF, as compared with the basal value (Fig. 5). However, these increases in GMBF were significantly reduced to 25.9% and 30.3% from 78.1% in the control group after 4 and 12 weeks treatment, respectively.

Effect of chronic treatment with TC or capsaicin pretreatment on CGRP release

The amount of CGRP released from the normal oxyntic mucosa after vehicle treatment (without capsaicin) was 1.12±0.12 pg/mg wet tissue. Incubation of the normal mucosa with capsaicin caused a significant increase in CGRP release of 113% (2.39±0.27 pg/mg wet tissue) (Fig. 6). In capsaicin pretreated rats, however, there was only a slight increase (13%) in CGRP release; the increase was significantly lower than the control values. One or two weeks after TC treatment, there was no significant change in CGRP release in response to capsaicin. However, CGRP release in response to capsaicin was only 35% (1.62±0.26 vs 1.20±0.17 pg/mg wet tissue in the basal value) after 4 weeks treatment with TC. This increase was significantly lower than that observed in the control value (without TC treatment).
Histological changes

Visible damage to the gastric mucosa of the control animals or of rats treated with TC for 1, 2, 4 or 12 weeks was not observed. Microscopical studies did not reveal any change in the mucosal thickness, parietal cell number, or proliferation of collagenous fibers and inflammatory cells by 2 weeks of TC treatment (Table 1, Fig. 7). After 4 weeks treatment, however, exfoliation of surface mucosal cells was observed in the majority of animals, without any changes in the above parameters. Twelve weeks later, a significant increase in inflammatory cell infiltration was observed in addition to the surface cell exfoliation. The structure of the glands was somewhat loose and irregular in the mucosa.

DISCUSSION

The present results clearly confirmed the well-known fact (20, 21) that a single topical application of TC to rat stomachs reduced PD followed by increases in luminal pH and GMBF. It has been shown that such GMBF

Fig. 3. Effects of 10 mM TC on PD and luminal pH in anesthetized rats pretreated with 10 mM TC for 1, 2, 4 or 12 weeks. A solution of 10 mM TC was topically applied to the stomachs for 10 min. Data are means ± 1 S.E. for 6 rats. *P < 0.05, significantly different from the control values, which were obtained 4 weeks after ingestion of drinking water alone. ○ Control, △ 1 week, □ 2 weeks, ▲ 4 weeks, ■ 12 weeks.

Fig. 4. Effect of 10 mM TC on GMBF in anesthetized rats pretreated with 10 mM TC for 1, 2, 4 or 12 weeks. A solution of 10 mM TC was topically applied to the stomachs for 10 min. Data are means ± 1 S.E. for 6 rats. *P < 0.05, significantly different from the control values, which were obtained 4 weeks after ingestion of drinking water alone. ○ Control, △ 1 week, □ 2 weeks, ▲ 4 weeks, ■ 12 weeks.
responses in the stomach after damage are mediated by capsaicin-sensitive neurons as well as endogenous prostaglandins (PGs) (22). We confirmed that the increase in GMBF caused by TC was significantly reduced in capsaicin-pretreated rats. The present study further demonstrated that chronic treatment with TC reduced the GMBF response to capsaicin as well as TC, suggesting the functional impairment of these sensory neurons. To note was that chronic treatment with TC did weaken the gastric mucosal integrity, resulting in the production of gastritis. These phenomena may contribute to desensitization of capsaicin-sensitive neurons.

In contrast to the acute experiments performed on normal animals, there is no data as to the effect of TC or capsaicin on the mucosal barrier or GMBF in animals chronically treated with TC for up to 12 weeks in relation to the production of gastritis. Of interest was that chronic treat-
ment with TC for more than 4 weeks produced a decrease in basal PD and an increase in luminal pH, suggesting continuous disruption of the mucosal barrier. More interesting was that the GMBF response to TC or even capsaicin was greatly reduced in these animals. The GMBF responses may be mediated by CGRP from the endings of capsaicin-sensitive neurons. It should be noted that while CGRP release in response to capsaicin was not affected after 1 or 2 weeks treatment with TC, it was significantly reduced after 4 weeks treatment with TC. These results strongly suggest that chronic treatment with TC gradually desensitizes capsaicin-sensitive neurons, which play an important role in the mucosal defense. However, the basal CGRP release following 4 weeks treatment with TC was very similar to that observed in the controls. These results suggest that while TC treatment for 4 weeks did not reduce the CGRP content in capsaicin-sensitive neurons, it lowers the responsiveness to capsaicin. The previous study showed that the increased GMBF response and the cytoprotective action induced by capsaicin was significantly suppressed by indomethacin, a cyclooxygenase inhibitor (7, 23). Endogenous PGs may be partly involved in the GMBF response to capsaicin. On the other hand, it has been reported that chronic treatment with TC caused a reduction in endogenous PGs in rats (24). It is possible to speculate that chronic treatment with TC reduced the endogenous PG, resulting in reduction of CGRP release from the nerve endings by capsaicin and the GMBF response to 10 mM TC or capsaicin.

Of note was that TC treatment for 4 weeks did not induce any microscopical change that suggested the production of gastritis. Szolcsanyi and Bartho (25) reported that the desensitization of capsaicin-sensitive neurons themselves did not affect the gastric secretory condition or the integrity of the gastric mucosa of rats. Their findings suggest that desensitization itself is not enough for the production of gastritis.

The exfoliation of surface epithelial cells and the infiltration of inflammatory cells are markers of gastritis. Such histological changes observed after 12 weeks TC treatment may be caused by continuous disruption of the mucosal barrier and the reduced GMBF caused by desen-
sitzation of capsaicin-sensitive neurons. Alternatively, it seems that the reduced GMBF on chronic treatment with TC cannot carry away the back-diffused acid from the irritated mucosa, which in turn results in the production of gastritis. These results suggest that the pathogenesis of reflux gastritis partly involves functional impairment of capsaicin-sensitive neurons by bile continuously refluxed into the stomach. Determination of the mucosal CGRP content in patients with reflux gastritis may provide a clue to the underlying mechanism. While we did not carry out any histological examinations, it is most likely that in the stomachs desensitized by subcutaneous capsaicin, the surface cell exfoliation occurred after 10 mM TC treatment.

We conclude that disruption of the gastric mucosal barrier and desensitization of capsaicin-sensitive neurons on chronic treatment with TC might greatly contribute to the pathogenesis of gastritis, primarily through the decreased GMBF.

Acknowledgments

We wish to thank Dr. K. Yamasaki (Otsuka Pharmaceutical Ltd., Tokushima) for the histological analysis, Dr. N. Inaba for determination of CGRP release and N.J. Halewood for critical reading of the manuscript.

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