Adaptive Gastric Cytoprotection Is Mediated by Prostaglandin EP1 Receptors: A Study Using Rats and Knockout Mice

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ABSTRACT
Endogenous prostaglandins (PGs) play a central role in adaptive cytoprotection induced in the stomach by mild irritants. In the present study, we used taurocholate (TC) as a mild irritant in both rats and EP-receptor knockout mice, and examined which EP receptor is responsible for the adaptive gastric cytoprotection. Gastric lesions were induced by p.o. administration of HCl/ethanol (60% ethanol in 150 mM HCl). TC (5–20 mM) or PGE2 was administered p.o. 30 min before HCl/ethanol. HCl/ethanol-induced gastric lesions were dose dependently prevented by TC, and the effect at 20 mM was equivalent to that induced by PGE2 at 0.3 mg/kg. The protective effect of TC was significantly attenuated by indomethacin as well as ONO-AE-829, the EP1 antagonist, but not by either NS-398, the selective cyclooxygenase (COX)-2 inhibitor, or chemical ablation of capsaicin-sensitive sensory neurons. Likewise, the protective action of PGE2 was also antagonized by ONO-AE-829 but not chemical deafferentation. TC significantly increased PGE2 contents in the stomach, with or without chemical deafferentation, and this effect was blocked in the presence of indomethacin but not NS-398 or ONO-AE-829. TC increased the mucosal PGE2 contents similarly in both wild-type and knockout mice lacking EP1 or EP3 receptors, yet the protective action of TC against HCl/ethanol was observed in both wild-type and EP3 receptor knockout mice, but not in mice lacking EP1 receptors. The present findings confirmed a role for endogenous PGE2 produced by COX-1 in adaptive gastric cytoprotection and suggested that this action is mediated by activation of EP1-receptors but not associated with capsaicin-sensitive afferent neurons.

It is known that a variety of prostaglandins (PGs) are capable of protecting the stomach against necrotizing agents, the phenomenon called gastric cytoprotection (Robert et al., 1979; Miller, 1983). Among them, E-type PGs and their derivatives are most effective in exhibiting cytoprotective action. In addition, it is known that a variety of mild irritants also exhibit gastric protection mediated by endogenous PGs, the phenomenon, called adaptive cytoprotection, that is, the response of the stomach induced by mild irritants to increase the mucosal resistance to injury (Miller, 1983; Robert et al., 1983).

On the other hand, the receptors activated by PGE2 are pharmacologically subdivided in at least four subtypes (EP1, EP2, EP3, and EP4) (Sugimoto et al., 1992; Watabe et al., 1993; Coleman et al., 1994). In situ hybridization study revealed that these EP receptors are all found in the stomach by in situ hybridization technique, especially EP1 in the muscularis mucosa; EP3 in the epithelial cell, parietal cell, and myenteric neurons; and EP4 in the epithelial cell, parietal cell as well as the mucus cell (Sugimoto et al., 1994; Morimoto et al., 1997). We have previously examined, using various prostanoids, subtype-specific EP receptor agonists and antagonist, the relationship of EP receptor subtypes, and PGE2-induced gastric cytoprotection in rats and found that this action was mediated by activation of EP1 receptors (Araki et al., 2000). However, it remains unexplored which EP receptor subtype is responsible for adaptive gastric cytoprotection induced by a mild irritant.

In the present study, we therefore investigated the EP receptor subtypes mediating adaptive cytoprotection induced by taurocholate (TC) in rat stomachs. The rationale to use TC as a mild irritant has been found in a previous study, showing that prior p.o. administration of taurocholate prevented gastric lesions in response to 0.6 N HCl (Takeuchi et al., 1995). Since an animal model lacking various receptors for prostanoids has been recently established (Sugimoto et al., 1997; Ushikubi et al., 1998), we also evaluated the adaptive cytoprotective activity of TC in knockout mice lacking EP1 or EP3 receptors.

Materials and Methods

Animals. Male Sprague-Dawley rats (200–220 g) and C57BL/6 mice (25–30 g) were used. Mice lacking the EP1 or EP3 receptors

ABBREVIATIONS: PG, prostaglandin; TC, taurocholate; COX, cyclooxygenase; EIA, enzyme immunoassay.
were generated as described previously (Sugimoto et al., 1997; Ushikubi et al., 1998). In brief, the mouse genes encoding the EP1 and EP3 receptors were individually disrupted, and chimeric mice were generated. These animals were then backcrossed with C57BL/6 mice, and the resulting heterozygous littermates were bred to produce homozygous EP1 or EP3 knockout mice. Distribution of the EP1 and EP3 receptor genes was verified by Northern blot hybridization, which failed to detect messenger RNAs encoding the respective receptors in EP1 (−/−) and EP3 (−/−) mice. These knockout mice and rats were deprived of food but allowed free access to tap water for 18 h before the experiments. All studies were performed using five to eight animals per group under unanesthetized conditions.

**Induction of Gastric Lesions.** The rats were given 1 ml of HCl/ethanol (60% in 150 mM HCl) p.o. through esophageal intubation, and killed 1 h later under deep ether anesthesia. The stomachs were removed, inflating by injecting 10 ml of 1% formalin for 10 min to fix the tissue walls, and opened along the greater curvature. The area (mm²) of hemorrhagic lesions developed in the stomach was measured using a dissecting microscope with a square grid (×10). TC (5–20 mM) was given p.o. as a mild irritant 30 min before administration of HCl/ethanol. PGE2 (0.03 mg/kg, i.v.) was given 10 or 30 min prior to HCl/ethanol treatment. In some cases, indomethacin (5 mg/kg), NS-398 (10 mg/kg), or ONO-AE-829 (5 and 10 mg/kg), the EP1 receptor antagonist (Watanabe et al., 1999), was given s.c. 1 h before administration of PGE2 or TC. In addition, the protective effect of PGE2 and TC against HCl/ethanol was also examined in rats with chemical ablation of capsaicin-sensitive sensory neurons (chemical deafferentation). Chemical deafferentation was induced by s.c. injections of capsaicin once daily for 3 consecutive days (total dose of 100 mg/kg) 2 weeks before the experiment (Takeuchi et al., 1994). All capsaicin injections were performed 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 injection. To check for the effectiveness of the treatment, a drop of capsaicin solution (0.1 mg/ml) was instilled into one eye of each rat, and the wiping movements were counted as previously reported (Holzer and Sametz, 1986).

In a separate experiment, wild-type mice and EP1 or EP3 receptor knockout mice were given HCl/ethanol orally in a volume of 0.3 ml, and killed 1 h later (Takeuchi et al., 1995). Then, the stomach was removed, treated with formalin, and the mucosa was examined for hemorrhagic lesions under a dissecting microscope, as described previously. In half the animals of each group, 20 mM TC was given p.o. 30 min before administration of HCl/ethanol. In addition, indomethacin (5 mg/kg), NS-398 (10 mg/kg), or ONO-AE-829 (10 mg/kg) was given s.c. 1 h before administration of TC in wild-type mice.

**Measurement of Mucosal Prostaglandin E2 Levels.** PGE2 levels in the gastric mucosa were measured 30 min after administration of 20 mM TC in rats and mice, including those lacking EP1 or EP3 receptors. In some cases, indomethacin (5 mg/kg), NS-398 (10 mg/kg), or ONO-AE-829 (10 mg/kg) was given s.c. 1 h before administration of TC. Under ether anesthesia, the stomachs were quickly removed, opened along the greater curvature, and rinsed with ice-cold saline. To separate the mucosal layer, the corpus mucosa was placed between two glass slides squeezed with a rubber band, and placed in hexane-frozen dry ice and acetone. These glasses were separated, and the mucosa was collected, weighed, and put in a tube containing 100% ethanol plus 0.1 M indomethacin (Putaki et al., 1994). Then, the samples were homogenized, and centrifuged for 10 min at 12,000 rpm at 4°C. The supernatant of each sample was used for determination of PGE2 by EIA using PGE2 kit (Cayman Chemical Co., Ann Arbor, MI).

**Preparation of Drugs.** Drugs used were PGE2 (Funakoshi, Tokyo, Japan), ONO-AE-829 (Ono, Osaka, Japan), taurocholate sodium (Difco, Detroit, MI), capsaicin (Nakarai Tesque, Kyoto, Japan), terbutaline (Bricanyl; Fujisawa, Osaka, Japan), aminophylline (Neophyllin; Eisai, Tokyo, Japan), and indomethacin (Sigma Chemical, St. Louis, MO). TC and ONO-AE-829 were dissolved in saline. PGE2 was first dissolved in absolute ethanol and then diluted with saline to a desired concentration. Capsaicin was dissolved in Tween 80-ethanol solution (10% ethanol, 10% Tween 80, 80% saline, w/w) for s.c. injection, whereas indomethacin was suspended in saline with a drop of Tween 80 (Wako, Osaka, Japan). Each agent was prepared immediately before use and given in a volume of 0.5 ml/100 g of body weight (rat) or 0.1 ml/10 g of body weight (mouse) in cases of p.o. and s.c. administration. Control animals received saline in place of active agent.

**Statistics.** Data are presented as the means ± S.E. from five to eight animals per group. Statistical analyses were performed using a two-tailed Dunnett’s multiple comparison test, and values of P < 0.05 were regarded as significant.

**Results**

**Protection by Exogenous PGE2 and TC against HCl/ Ethanol-Induced Gastric Lesions.** Oral administration of ethanol (60% in 150 mM HCl) produced multiple lesions in the glandular mucosa, along the long axis of the stomach, the lesion score being 108.6 ± 7.2 mm². These lesions were potently prevented by prior i.v. administration of PGE2 (0.03 mg/kg), the inhibition being 82.1% (Fig. 1). The protective effect of PGE2 was mitigated dose dependently by pretreatment with the EP1 antagonist ONO-AE-829 (5 and 10 mg/kg) but not by chemical deafferentation. The degree of protection afforded by PGE2 in the presence of ONO-AE-829 at 10 mg/kg was 19.8%, which is significantly less than that observed in normal rats. Likewise, the severity of HCl/ethanol-induced gastric lesions was dose dependently reduced in the animals pretreated with TC (5–20 mM) p.o. before challenge with HCl/ethanol, and a significant effect was obtained at over 10 mM, the inhibition at 20 mM TC being 92.4% (Fig. 2). The protective action of TC (20 mM) was almost totally attenuated by prior administration of ONO-AE-829 (10 mg/kg) as well as indomethacin (5 mg/kg), but not by either NS-398 (10 mg/kg) or chemical deafferentation.

**Mucosal PGE2 Contents in the Stomach Exposed to TC.** Levels of PGE2 in the normal rat gastric mucosa were 41.31 ± 7.6 ng/g of tissue. Oral administration of 20 mM TC

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significantly increased the PGE2 levels in the corpus mucosa of normal rats when determined at 0.5 h after the administration, the levels being 230.6 ± 27.3 ng/g of tissue (Fig. 3).

Prior administration of indomethacin (5 mg/kg s.c.) markedly reduced the enhanced PG generation in the gastric mucosa following TC treatment, the inhibition being 80.7%. By contrast, neither NS-398 (10 mg/kg), nor ONO-AE-829 (10 mg/kg) had any effect on the increased PGE2 response caused in the stomach by TC treatment. The enhanced PG generation by TC was similarly observed in chemically deafferented rats following capsaicin pretreatment, the levels being 228.6 ± 14.3 ng/g of tissue. The increase of mucosal PGE2 response to TC in these animals was also totally blocked in the presence of indomethacin.

Gastric Cytoprotection by TC in Mice against HCl/Ethanol. Intragastric administration of HCl/ethanol (0.3 ml) also provoked hemorrhagic lesions in the mouse stomach, the lesion score being 24.3 ± 6.9 mm² (Fig. 4). Similar to the findings in rats, the severity of these lesions was reduced by prior p.o. administration of TC (5–20 mM), in a dose-dependent manner, although a significant effect was observed only at 20 mM, the inhibition being 80.2%.

To further investigate the relationship of TC-induced gastroprotection and EP receptor subtypes, we examined the protective effect of TC against HCl/ethanol in both wild-type and knockout mice lacking EP1 or EP3 receptors, as well as the reversal by indomethacin and ONO-AE-829, the EP1 antagonist. As shown in Fig. 5, HCl/ethanol caused gastric lesions in both EP1 and EP3 receptor knockout mice, similar to wild-type mice, and the severity of these lesions was about same among these groups. Twenty micromolar TC given p.o. 30 min before HCl/ethanol significantly reduced the severity of these lesions in both wild-type and EP3 receptor knockout mice, but the effect totally disappeared in the animals lacking EP1 receptors; the degree of protection being 92.6, 94.1, and 27.3%, respectively, in wild-type, EP3, and EP1 receptor knockout mice. The protective action of TC against HCl/Ethanol-induced gastric lesions in rats. The animals were given 1 ml of HCl/ethanol (60% ethanol in 150 mM HCl) p.o. and killed 1 h later. TC (1 ml: 5–20 mM) was given p.o. 30 min before HCl/ethanol. Data are presented as the means ± S.E. from four rats. *Statistically significant difference from control at P < 0.05. B, effects of indomethacin, ONO-AE-829, and chemical deafferentation on the protective action of 20 mM TC against HCl/ethanol-induced gastric lesions in rats. Indomethacin (5 mg/kg), NS-398 (10 mg/kg), and ONO-AE-829 (10 mg/kg) were given s.c. 1 h before administration of 20 mM TC, whereas chemical deafferentation was induced by s.c. injection of capsaicin once daily for 3 consecutive days (total of 100 mg/kg) 2 weeks before the experiment. Data are presented as the means ± S.E. from six to eight rats. Statistically significant difference at P < 0.05; *from control in the corresponding group; #from vehicle.
mice. Statistically significant difference at P < 0.05; *from control in the corresponding group; †from vehicle.

Fig. 5. Effects of 20 mM TC on HCl/ethanol-induced gastric lesions in wild-type and knockout mice lacking EP1 or EP3 receptors. The animals were given 0.3 ml of HCl/ethanol (60% ethanol in 150 mM HCl) p.o. and killed 1 h later. TC (0.3 ml: 20 mM) was given p.o. 30 min before HCl/ethanol. In wild-type mice, indomethacin (5 mg/kg), NS-398 (10 mg/kg), and ONO-AE-829 (10 mg/kg) were given s.c. 1 h before administration of TC. Data are presented as the means ± S.E. from four to five mice. Statistically significant difference at P < 0.05; *from control in the corresponding group; †from vehicle.

The mucosal PGE2 generation was also increased by intragastric administration of 20 mM TC in mice, irrespective of whether EP1 or EP3 receptors were knocked out, the degree of increase being similar among these groups (Fig. 6). The increased PGE2 response induced in wild-type mice by TC was significantly inhibited by prior administration of indomethacin (5 mg/kg), but not by NS-398 (10 mg/kg).

Fig. 6. PGE2 generation in the gastric mucosa after oral administration of TC in wild-type and knockout mice, lacking EP1 or EP3 receptors. The animals were given 20 mM TC (0.3 ml) p.o., killed 0.5 h later, and the mucosal PGE2 contents were determined by EIA. In wild-type mice, indomethacin (5 mg/kg) or NS-398 (10 mg/kg) was given s.c. 1 h before administration of TC. Data are presented as the means ± S.E. from five mice. Statistically significant difference at P < 0.05; *from control in the corresponding group; †from vehicle.

Discussion

PGs, either endogenous or exogenous derivatives, act on multiple receptors (Coleman et al., 1994). In a previous study, we investigated the relationship of EP receptor subtypes and gastric protection against HCl/ethanol in rats using various EP agonists and found that exogenous PGE2 afforded gastric cytoprotection mediated by EP1 receptors (Araki et al., 2000). On the other hand, it is known that a variety of mild irritants also exhibit gastric protection mediated by endogenous PGs, the phenomenon, called adaptive cytoprotection (Miller, 1982; Robert et al., 1983). However, it remains unexplored which EP receptor subtype is responsible for this phenomenon. The present study showed using rats and EP receptor knockout mice that the adaptive cytoprotection induced in the stomach by a mild irritant is mediated by endogenous PGE2 through activation of EP1 receptors.

First, we confirmed that exogenous PGE2 given i.v. potentially prevented the development of HCl/ethanol-induced gastric lesions, and this action was significantly attenuated by the EP1 antagonist ONO-AE-829 (Araki et al., 2000). These results are in agreement with our previous observations using various EP agonists in rats (Araki et al., 2000). In that study, we showed that these lesions were prevented by prostanooids specific to EP1 receptors such as 17-phenyl PGE2 and sulprostone but not other types of EP agonists, including butaprost (EP2 agonist), ONO-NT-012 (EP3 agonist), or 11-deoxy PGE1 (EP4 agonist). This contention was also confirmed in EP receptor knockout mice, showing that the PGE2 protection disappeared in the mice lacking EP1 receptors (Araki et al., 2000). Thus, the present together with previous data strongly suggest that the protective action of exogenous PGE2 in the stomach is mainly mediated by activation of EP1 receptors.

Second, it is known that endogenous PGs also mediate adaptive cytoprotection observed in the stomach pre-exposed to mild irritants (Miller, 1983; Robert et al., 1983). In the present study, we used 20 mM TC as a mild irritant and showed that this agent increased PGE2 generation in the stomach and inhibited the occurrence of damage in response to HCl/ethanol. These changes caused by TC totally disappeared in the presence of indomethacin, confirming the involvement of endogenous PGE2 in this phenomenon. Of interest, the protective effect of TC was also attenuated by ONO-AE-829, the EP1 antagonist, similar to that afforded by exogenous PGE2. Since this agent did not affect the increased formation of PGE2 in the stomach after TC treatment, it is considered that the reversal by this agent of TC-induced gastric protection occurs through antagonism at the EP1 receptors. Indeed, we observed in EP receptor knockout mice that TC failed to show gastric protection against HCl/ethanol in mice lacking EP1 receptors, although this agent exhibited a potent protection against these lesions in both wild-type and EP3 receptor knockout mice. Since TC increased PGE2 generation in the stomach by the same degree, irrespective of whether EP1 or EP3 receptors were knocked out, it is likely that the absence of TC-induced gastric protection is due to a lack of EP1 receptors in these animals. These data strongly suggest that mild irritants induce adaptive gastric protection, mediated by endogenous PGE2 through activation of EP1 receptors.

A number of studies have been conducted to clarify the mechanisms involved in adaptive protection in the stomach following mild irritants (Takeuchi et al., 1987; Mercer et al., 1988; Matsumoto et al., 1991), yet the exact mechanism remains unknown. Since this phenomenon is mediated by endogenous PGE2, as shown in the present study, the mechanisms should be related to the actions reproduced by exog-
enous PGE2. It is known that PGE2 causes various changes in gastric functions, including an inhibition of acid secretion, an increase of mucus/bicarbonate secretion, an increase of gastric mucosal blood flow, and inhibition of gastric motility (Miller, 1983). Among these actions, the effect on acid or bicarbonate secretion can be excluded in the possible mechanisms of TC-induced protection, because gastric lesions in the present study were induced by ethanol plus 150 mM HCl, masking changes in endogenous acid secretion. We previously reported that PGE2 caused inhibition of gastric motility as well as increase of mucosal blood flow and mucus secretion, and these effects were mimicked by different EP agonists, i.e., these effects were reproduced by prostanoids activating EP1 receptors, EP2 and EP3 receptors, and EP4 receptors, respectively (Takeuchi et al., 1997; Araki et al., 2000). Since TC-induced gastric protection was attenuated by the EP1 antagonist and since this phenomenon disappeared in the mice lacking EP1 receptors, it is assumed that adaptive gastric protection against HCl/ethanol may be functionally associated with inhibition of gastric motility, similar to the protection induced by exogenous PGE2 (Araki et al., 2000). Although we did not examine the effect of TC on gastric motility in the present study, it has been reported that a variety of compounds, including mild irritants, inhibited gastric motility at their cytoprotective doses (Takeuchi et al., 1988, 1989, 1992). Further studies should certainly be needed on this point.

It should be noted in the present study that the protective effect of exogenous PGE2 against HCl/ethanol was not affected by chemical ablation of capsaicin-sensitive sensory neurons. Likewise, TC-induced gastric protection was also not affected by chemical deafferentation. These observations are controversial with previous findings by others that sensory deafferentation significantly attenuated the protective effect of PGE2 or mild irritants against ethanol (Mercer et al., 1988; Esplugues et al., 1992). Although the reason for this controversy remains unknown, it may be due to different experimental conditions, including the period of fasting: the strain of rats; or the environmental factors such as breeding, housing, and chow. The effect of chemical deafferentation might appear differently, depending on the tonus of the sensory neurons; if it were maintained in “tonic conditions”, then the chemical deafferentation would negatively affect the protective action of mild irritants.

On the other hand, there are two isozymes for the enzyme producing PGs, that is, cyclooxygenase (COX)-1 and COX-2 (Feng et al., 1993). Although COX-1 accounts for the majority of PG synthesis in the normal stomach, recent studies suggest that COX-2-derived PGs also play a role in the maintenance of gastric mucosal integrity (Gretzer et al., 1998; Konturek et al., 1998; Brzozowski et al., 2000). We also found that COX-2 was up-regulated in the stomach from 3 h after TC treatment and was involved in the later period of TC-induced gastric protection (Yamamoto et al., 1999). Gretzer et al. (1998) even showed that the selective COX-2 inhibitors such as NS-398 or L-745,337 abolished the early protective effect of 20% ethanol against gastric damage in response to 96% ethanol and suggested that PGs generated by COX-2 could contribute to physiological functions involved in gastric homeostasis. In their study, the luminal exposure to 20% ethanol did not result in a measurable increase in PG formation, and the COX-2 inhibitors failed to affect the mucosal PG generation in the stomach pre-exposed to 20% ethanol. In the present study, however, NS-398, the selective COX-2 inhibitor, did not affect both the increased PGE2 production and the protective action against HCl/ethanol observed 1 h after TC treatment in both rats and mice. The reason for the discrepancy between these two studies remains unexplained, yet the former study cannot exclude the possibility that effects other than suppression of PG production mediate the attenuation of 20% ethanol-induced protection elicited by the selective COX-2 inhibitors. Thus, it is likely that TC-induced gastric protection observed in this study is mediated by PGE2 produced by COX-1 but not COX-2. Indeed, we previously reported that 20 mM TC increased PGE2 production in rat stomachs when determined 30 min after the administration, and that this PG biosynthetic response was inhibited by indomethacin, but not NS-398 (Hirata et al., 1997; Yamamoto et al., 1999).

Taken together, the present study confirmed a critical role for endogenous PGE2 in adaptive cytoprotection induced in the stomach by a mild irritant and suggested that this action is mediated by PGE2 through EP1 receptors but is not associated with capsaicin-sensitive afferent neurons. It is concluded that PGE2, either generated endogenously or administered exogenously, exhibits gastric protection by activation of EP1 receptors. Further studies should be needed to clarify the functional mechanism for this phenomenon, in relation to EP receptor subtypes.

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