Modification of 5-Hydroxytryptophan-Evoked 5-Hydroxytryptamine Formation of Guinea Pig Colonic Mucosa by Reactive Oxygen Species

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ABSTRACT—We studied whether reactive oxygen species (ROS) generated by normal colonic mucosa affect 5-hydroxytryptophan (5-HTP)-evoked 5-HT formation (measured as the sum of 5-HT plus 5-hydroxyindole acetic acid (5-HIAA) accumulation) of guinea pig’s isolated colonic mucosa. Catalase (3000 – 6000 U/ml), a hydrogen peroxide (H_2O_2) scavenger or diphenylene iodonium (DPI, 10 – 100 μM), an NADPH oxidase inhibitor, concentration-dependently caused an increase of the sum of 5-HT plus 5-HIAA accumulation in the presence of 5-HTP (10 μM), but these drugs did not significantly affect the 5-HT-metabolite in the colonic mucosa measured as the ratio of 5-HIAA/5-HT. Exogenously applied H_2O_2 (10 – 100 μM) concentration-dependently inhibited the sum of 5-HT plus 5-HIAA accumulation. In contrast, neither superoxide dismutase (SOD, 100 – 300 U/ml), superoxide anion scavenger, nor dimethyl sulfoxide (1 – 5%, DMSO), a hydroxyl radical scavenger affected the sum of 5-HT plus 5-HIAA accumulation. Moreover, mucosa ROS generation was estimated using the chemiluminescence technique. SOD (100 – 300 U/ml), catalase (3000 – 6000 U/ml) or DPI (10 – 100 μM), concentration-dependently reduced luminol-enhanced chemiluminescence signal from the colonic mucosa, while allopurinol (10 – 100 μM), a xanthine oxidase inhibitor, did not affect the chemiluminescence signal. These results suggest that ROS is formed through an NADPH oxidase system in the guinea pig colonic mucosa, where it exerts a modulatory effect on mucosal 5-HT formation upon addition of 5-HTP. Thus, ROS formation from normal colonic mucosa could be considered to contribute to the control of 5-HT production in mucosa enterochromaffin cells.

Keywords: 5-Hydroxytryptamine, Colon (guinea pig), Enterochromaffin cell, 5-Hydroxytryptophan, Reactive oxygen species

5-Hydroxytryptamine (5-HT) has long been recognized as an important messenger substance involved in a wide variety of intestinal functions exerted through its interaction with multiple receptor subtypes (1), and in functional bowel disorders (2). Most of the intestinal 5-HT is produced and stored in mucosal enterochromaffin cells (EC cells) (3, 4) from which this amine is released into the intestinal lumen and the portal circulation (5, 6). The regulatory mechanism of 5-HT release from the EC cells has been studied more extensively, using a variety of experimental models in different animals (7 – 9), but the regulation of 5-HT production in the EC cells is not fully understood.

Reactive oxygen species (ROS), such as superoxide anion (O_2·⁻) and hydrogen peroxide (H_2O_2), are thought to be involved in the pathogenesis of experimental colitis in animal models and inflammatory bowel disease of humans (10 – 12), and in the defense against invading foreign organisms (13). The free radicals are released from various types of cells residing in intestinal mucosa. In this respect, neutrophils, as well as epithelial cells are known to be potent sources of these ROS (14). Normal colonic mucosa is also shown to be capable of producing ROS (15, 16), but whether ROS produced by the normal colonic mucosa affect 5-HT production in the EC cells, is currently unclear. More recently, we have shown that the colonic mucosa of guinea pig is capable of converting the 5-HT precursor 5-hydroxytryptophan (5-HTP) to 5-HT (17). Therefore, the aim of the present study was to provide evidence for understanding the action of ROS on 5-HT production in colonic mucosa EC cells by elucidating the effects of ROS scavengers on 5-HTP-evoked 5-HT formation of guinea pig colonic mucosa.
MATERIALS AND METHODS

Tissue preparation

Male Dunkin-Hartley guinea pigs of 250 – 300 g body weight were purchased from Shizuoka Laboratory Animal Center, Inc. (Shizuoka). Guinea pigs were anesthetized with enflurane and bled via the femoral artery. A segment of the proximal colon, 2 – 9.5-cm away from the caecum was removed and the longitudinal/circular muscle layers were carefully stripped off as previously described (18). The underlying mucosa sheet and muscle layers were suspended in 15-ml tissue baths filled with modified Krebs solution (120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11 mM glucose and 0.03 mM Na₂EDTA) at 37°C and bubbled with 95% O₂ / 5% CO₂. The tissues were then washed in modified Krebs solution for 60 min before the start of experiments. During the washing period, the bathing medium was replaced every 10 min. The colonic mucosa was subsequently divided longitudinally in 7 – 9 pieces for biochemical experiments.

Measurement of 5-HT and 5-hydroxyindole acetic acid (5-HIAA)

The mucosa pieces (approximately 20 – 50 mg each) and muscle strips (1-cm-long) were suspended in 1.9-ml tissue baths containing preoxygenated Hank’s balanced salt solution (HBSS, pH 7.4) kept at 37°C. The HBSS mostly contained tetrodotoxin (1 μM), a nerve conduction blocking agent and pyridoxal (1 μM), a decarboxylase cofactor. After 5 min of preincubation, 5-HTP (10 – 100 μM) was added into the medium. When the effects of scavengers/inhibitor or vehicle at 37°C. After 10 min of preincubation, the test tubes were placed in the luminescence reader (BLR-301; Aloka, Tokyo), and luminol (100 μM) was added into the medium prior to measurement of chemiluminescence for 3 min at 37°C. Chemiluminescence is expressed as a percentage of the control after subtraction of background (mean of photon counts detected by a test tube containing luminol and HBSS before and after each measurement from tissue suspension).

Drugs

The following drugs were used: tetrodotoxin (Sankyo, Tokyo); pyridoxal hydrochloride (Wako Pure Chemical, Osaka); benserazide hydrochloride (Research Biochemicals Inc., Natick, MA, USA); allopurinol, catalase, dimethyl sulfoxide, diphenylene iodonium (DPI), H₂O₂, 5-hydroxytryptophan, luminol, pyrogallol, superoxide dismutase (SOD) (Sigma Chemical Co., St. Louis, MO, USA). All drugs were initially dissolved in saline with the exception of luminol (20 mM), which was dissolved in 0.1 M NaOH.

Statistical analyses

Results are expressed as means ± S.E.M. Statistical analysis was performed by ANOVA, followed by post hoc evaluation (according to Bonferroni or Dunnett where appropriate). A P value <0.05 was considered significant.

RESULTS

5-HTP-evoked 5-HT accumulation

In the presence of tetrodotoxin (1 μM) and pyridoxal (1 μM), increasing concentration of 5-HTP (90-min treatment) in the incubation medium resulted in a concentration-dependent increase in the 5-HT accumulation of isolated colonic mucosa (Fig. 1). Furthermore, the increase in the 5-HT accumulation in response to the highest concentration of 5-HTP used (100 μM) was inhibited by coincubation of tissues with the amino acid decarboxylase inhibitor benzazide (30 μM) (Fig. 1). In contrast, 5-HTP (100 μM) was a poor amplifier of the 5-HT accumulation of the longitudinal/circular muscle preparations in the presence of pyridoxal (0.16 ± 0.07 nmol/g tissue, n = 5, Fig. 1).

Effects of scavengers/inhibitor

To evaluate the possible effects of ROS on 5-HTP (10 μM)-evoked 5-HT formation (measured as the sum of 5-HT plus 5-HIAA accumulation), ROS scavengers and inhibitor were tested. After 90 min of incubation with 5-HTP (10 μM), accumulation levels of 5-HT and the 5-HT metabolite 5-HIAA was 6.0 ± 0.6 nmol/g tissue and 0.8 ± 0.2 nmol/g tissue (n = 9), respectively. As shown in Fig. 2A, catalase (3000 – 6000 U/ml), H₂O₂ scavenger or DPI (10 – 100 μM), an NADPH oxidase inhibitor, concen-
5-hydroxytryptophan (5-HTP, 10–100 μM)-evoked 5-HT accumulation (90 min) of guinea pig colonic mucosa in the presence of tetrodotoxin (1 μM) and pyridoxal (1 μM), a decarboxylase cofactor. Some experiments were performed with the addition of the decarboxylase inhibitor benserazide (BEZ, 30 μM) or using longitudinal/circular muscle preparations (LCM) in the absence of tetrodotoxin. The 5-HTP-evoked 5-HT accumulation is expressed as nanomole per gram wet weight of tissue. Each column represents the mean and vertical lines show the S.E.M. of 5–9 experiments. **P<0.01 vs the control, *P<0.05 vs the 5-HTP (100 μM) alone.

Chemiluminescence studies

The mucosa pieces suspended in buffer produced a measurable chemiluminescence signal. SOD (100–300 U/ml), catalase (3000–6000 U/ml) or DPI (10–100 μM) concentration-dependently reduced the luminol-enhanced chemiluminescence signal from the colonic mucosa (Fig. 3). In contrast, allopurinol (10–100 μM), a xanthine oxidase inhibitor, did not affect the chemiluminescence signal (Fig. 3). Longitudinal/circular muscle preparations did not produce a chemiluminescence signal (n = 4, data not shown).

DISCUSSION

The present study deals with the question of whether ROS generated by the colonic mucosa affect mucosal 5-HT formation upon addition of 5-HTP, the endogenous 5-HT precursor. Under the present in vitro conditions (in the...
5-HTP-evoked 5-HT formation and ROS

Effects of ROS scavengers/inhibitor on luminol (100 μM)-enhanced chemiluminescence from the colonic mucosa.

**Fig. 3.**

| Drug | Chemiluminescence (%) |
|------|------------------------|
| CONT |                        |
| SOD 100 |          |
| SOD 300 |          |
| CAT 3000 |         |
| CAT 6000 |          |
| DPI 10 | **          |
| DPI 100 | **          |
| ALO 10 | **          |
| ALO 100 | **         |

Each column represents the mean and the vertical line shows the S.E.M. of 5–7 experiments. **P<0.01 vs the control.

The presence of tetrodotoxin, 5-HTP concentration-dependently increased the 5-HT accumulation in the isolated guinea pig colonic mucosa, which closely correlates with changes in the 5-HTP-evoked luminal 5-HT release from the colon (17), whereas 5-HTP was a poor amplifier of the 5-HT accumulation of the longitudinal/circular muscle preparations with adherent myenteric plexus. Thus, these results suggest that 5-HTP-evoked 5-HT accumulation in this preparation reflects almost exclusively 5-HT production from non-neuronal 5-HT-producing cells in the colonic mucosa. This conclusion is in keeping with the notion that no 5-HT-immunoreactive neurons in the guinea pig colonic mucosa is found (20). The EC cells have commonly been addressed, as a candidate of the 5-HT-producing cells in the colonic mucosa. In addition, we recently reported (17) that using the luminally perfused isolated guinea pig colon, 5-HTP facilitates the luminal 5-HT release from EC cells. Thus, it is possible that the 5-HTP-evoked 5-HT formation occurs in EC cells, although the amount of the 5-HTP-evoked 5-HT formation in part contains that due to “epithelial cells” contamination (7). If 5-HT is produced also by the epithelial cells, the function of that 5-HT compared to that produced by the EC cells is largely unknown.

To determine the influence of ROS on the enhanced 5-HT formation by 5-HTP, we examined the effects of catalase, a H₂O₂ scavenger; SOD, O₂⁻ scavenger; and DMSO, a hydroxyl radical scavenger. In this study, we found that 5-HT formation (measured as the sum of 5-HT plus 5-HIAA accumulation) of the colonic mucosa evoked by 5-HTP was enhanced in the presence of catalase, suggesting a role for hydrogen peroxide as a modulator of 5-HT biosynthesis. Furthermore, the possibility that H₂O₂ may affect 5-HT degradation processes is unlikely, because catalase had no effect on 5-HT-metabolism (measured as the ratio between 5-HIAA/5-HT). In contrast, SOD and DMSO failed to modify the 5-HT formation in response to 5-HTP. The potency of SOD or DMSO used in the present study has been well documented (10, 12, 21). Thus, it seems likely that neither O₂⁻ nor a H₂O₂ hydrogen peroxide metabolite, hydroxyl radical, participated in the modification of the enhanced 5-HT formation.

Furthermore, we have examined whether ROS are generated from the colonic mucosa using the chemiluminescence technique. In the present study, we showed that the colonic mucosa fragments could produce a measurable luminol-enhanced chemiluminescence signal. The marked decrease in chemiluminescence signal caused by SOD and the NADPH oxidase inhibitor DPI suggests that superoxide anion predominantly via an NADPH oxidase system is generated from the colonic mucosa. The partial decrease in the chemiluminescence signal caused by catalase also suggests that H₂O₂ is one of the ROS detected in the colonic mucosa. These findings are consistent with previous data showing that the normal rat colonic mucosa (15) or the normal guinea pig gastric mucosa (22) spontaneously generates superoxide anion. Nevertheless, SOD failed to modify the enhanced 5-HT formation by 5-HTP. Such data implies that O₂⁻ may be only useful as a precursor of the more stable ROS, H₂O₂. In the present study, we also observed that increasing concentrations (10–100 μM) of H₂O₂ in the incubation medium resulted in a decrease in the enhanced 5-HT production. Hence, it is considered that the enhancement of the 5-HT formation to 5-HTP by catalase is due to the degradation of the enhanced extracellular H₂O₂, resulting from the dismutation of superoxide anion. There are various possible sources of ROS production in the colonic mucosa; they include xanthine oxidase within epithelial cells and NADPH oxidase in phagocytic leukocytes (23). In our experiments, xanthine oxidase does not contribute substantially to the ROS production as evidenced by a lack of effect of allopurinol, a xanthine oxidase inhibitor, on the chemiluminescence signal. Moreover, it is well known that neutrophils are abundantly present in the inflamed colonic mucosa, but not in the normal colonic mucosa (10, 16). In accordance with the effect of catalase, DPI also enhanced the 5-HT formation evoked by 5-HTP. This probably represents the role of NADPH oxidase system in regulating 5-HT levels of the colonic mucosa. Recently Suh et al. (24) showed that the superoxide-generating NADPH oxidase...
Mox1 messenger RNA is expressed most in normal human colon, but not in peripheral blood leukocytes. Thus, a superoxide-generating NADPH oxidase Mox1-like system might contribute to the superoxide generation from the colonic mucosa, as a possible source.

In conclusion, the results of the present study provide evidence that ROS is formed through an NADPH oxidase system in the guinea-pig colonic mucosa, where it exerts a modulatory effect on mucosal 5-HT formation upon addition of 5-HTP. Thus, ROS formation from normal colonic mucosa could be considered to contribute to the control of 5-HT production in mucosa enterochromaffin cells.

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