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Interactions of EGF and Ornithine Decarboxylase Activity in the Regulation of Gastric Mucus Synthesis in Cigarette Smoke Exposed Rats

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Abstract

Cigarette smoking has been shown to aggravate ulceration and delay ulcer healing. Smokers had a lower level of mucus in their stomachs. In the present study, we examined whether cigarette smoke or its extract reduced mucus production through the suppression of epidermal growth factor (EGF) associated with the reduction of polyamine biosynthesis both *in vivo* and *in vitro*. Ornithine decarboxylase (ODC) activities and mucus synthesis were determined in rat gastric mucosa and in human MKN-28 cells. Incubation of MKN-28 cells with EGF (0.01-1.00 ng/mL) significantly increased mucus synthesis in vitro, which was accompanied by an increase of ODC activity. Removal of salivary glands decreased the circulated EGF level and induced a significant reduction of mucus-secreting layer thickness in the gastric mucosa. Cigarette smoke or its extract markedly decreased mucus synthesis in vivo and in vitro, both of which could be completely reversed by intravenous administration of EGF (20 μg/kg) in rats or co-incubation with EGF (1 and 2 ng/mL) in MKN-28 cells. However, ODC activities, which were suppressed by cigarette smoke or its extract, were unaffected by intravenous administration of EGF in rats, or only partially reversed by co-incubation with EGF in MKN-28 cells. These findings indicate that both EGF and ODC activity represent two different entities in the modulation of cigarette smoking on gastric mucus synthesis. The action of EGF on mucus synthesis may only be partially if not dependent on ODC activity in the stomach.

Key Words: cigarette smoking, gastric mucus, epidermal growth factor, ornithine decarboxylase

Introduction

Apart from HIV/AIDS, tobacco smoking is another major cause of death, which is increasing rapidly (1). China, with 20 percent of the world's population, produces and consumes about 30 percent of the world's cigarettes (19). China already suffers almost a million deaths a year from tobacco smoking (15). There are number of diseases which have a causal relationship with smoking. Basic and clinical studies showed that cigarette smoking is associated with the occurrence and recurrence of peptic ulcer diseases, and delays ulcer healing. However, the underlying mechanisms are obscure.

Adherent mucus gel provides a physical barrier and a stable unstirred layer between the apical surfaces of the epithelial cells and the lumen. Mucus-bicarbonate establishes a pH gradient, which protects against acid and pepsin invasion. Furthermore, increased mucus at the site of mucosal injury enhances the binding of EGF and other growth factors to the corresponding receptors, resulting in the augmentation of cell proliferation (23). Apparently, mucus is important in mucosal protection and ulcer healing.

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Epidermal growth factor (EGF) is a 53 amino acid polypeptide, which stimulates mRNA, DNA and protein synthesis mainly in epithelial cells (5). EGF has an ideal spectrum of biological activities to protect mucosa against injury and facilitate mucosal repair and proliferation in the gastrointestinal tract (26). It has been shown that EGF stimulates mucus synthesis and secretion from biopsies of human antral mucosa (13). In addition, smokers have lower levels of salivary EGF (14) and acid mucosubstances in the gastric epithelium than non-smokers (10). A number of studies demonstrated that polyamines are involved in EGF-mediated gastroprotection, ulcer healing and inhibition of acid secretion (18, 25). Ornithine decarboxlyase (ODC) is the first rate-limiting enzyme in the biosynthesis of polyamine. Yet, little is known about the effect of cigarette smoking on gastric mucosal ODC activity and the interaction between EGF and ODC in mucus synthesis. We, therefore, attested the hypothesis that whether cigarette smoking could reduce mucus synthesis in the gastric mucosa through EGF and ODC pathways.

Materials and Methods

Chemicals and Drugs

Chemicals and drugs were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. EGF was dissolved in sterile normal saline. In animal study, EGF (20 μg/kg) was administered intravenously once daily immediately prior to smoke exposure. In cell culture, EGF (0.01-1.00 ng/mL) was incubated in MKN-28 cells for 6 h.

Animals

Male Sprague-Dawley rats (180-200 g) were reared with rodent chow (Ralston Purina Co., Chicago, IL, USA) and given tap water. They were kept in a room where temperature (22±1°C), humidity (65-70%), and day/night cycle (12h/12h) were maintained.

Cigarette Smoke Exposure

Non-filtered cigarettes (Camel, 1.2 mg of nicotine and 18 mg of tar per cigarette, R.J. Reynolds, Winston-Salem, NC, USA) were used throughout the study. Rats were exposed to cigarette smoke (4%, vol/vol) in a chamber for a 1-h period once daily for 3 or 6 days. Detailed procedures for cigarette smoke exposure and equipment used were described previously (6). It was shown that smoke exposure did not affect the normal physiological functions of rats such as acid/base balance, and O_2/CO_2 in blood, heart rate and blood pressure. Twenty-four hours after the final cigarette smoke exposure, rats were killed by cervical dislocation. The stomachs were removed and opened along the greater curvature. A longitudinal section of the stomach along the greater curvature was taken and fixed in 4% buffered formalin for 24 h at 4°C for histological study. The remaining glandular mucosa was scraped with a glass slide on an ice-cold dish and immediately frozen in liquid nitrogen. The mucosal samples were stored at -70°C until assay.

Extraction of Substances from Cigarette Smoke

The substances in cigarette smoke were extracted by pumping into a series of bottles containing 500 mL of chloroform. The substances in the smoke were absorbed in the solvent. The chloroform fraction was collected and concentrated by a rotary evaporator (Yamato Scientific Co. Ltd., Tokyo, Japan). Our previous study showed that the chloroform fraction potentiated ethanol-induced gastric ulceration (7). Therefore, this fraction was used to examine the effect of cigarette smoke components on mucus synthesis in MKN-28 cells.

Determination of Mucus Synthesis in MKN-28 Cells

MKN-28 is a secretory type of human gastric carcinoma cell line, which was derived from a moderately-differentiated tubular adenocarcinoma (9). The cells (1×10^5 cells/0.5 mL of medium) were incubated at 37°C in a 24-well culture plate (Costar Corporation, Cambridge, MA, USA) in a humidified, 5% CO_2 atmosphere, in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (GibcoBRL, Grand Island, NY), 0.2 g/L streptomycin, and 0.1 g/L penicillin. The rate of mucus synthesis was determined by measuring the incorporation of D-[6-^{3}H] glucosamine into gastric mucosal glycoprotein according to the method of Terano et al. (24). When cells reached a confluence after an overnight incubation, they were washed twice with Ca^{++}-, Mg^{++}-free PBS, followed by incubation with 0.5 mL of the medium containing [^{3}H]glucosamine HCl (Amersham, Little Chalfont, UK), cigarette extract or its vehicle at 37°C, 5% CO_2 for 6 h. At the end of incubation, medium was aspirated. The remaining cells were washed twice with Ca^{++}, Mg^{++} free PBS, solubilized with 0.4 mL of 0.3 mol/L NaOH and neutralized with 0.4 mL of 0.3 mol/L HCl. Then the radioactivity of the acid insoluble fraction in the resulting aliquot was mixed with 9 mL of scintillation fluid and counted in a liquid scintillation counter (LS-6500; Beckman Instruments, Fullerton, CA, USA). Mucus synthesis was expressed as the ratio of [3H]glucosamine incorporation to the control group.

Estimation of Cell Viability

Cell viability was estimated using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (17). The cells (2×10^4/100 μL medium) were seeded into a 96-well plate and incubated overnight for attachment. They were then incubated with EGF, cigarette extract or its vehicle, at 37°C for 6 h. At the end of incubation, the medium was aspirated. The remaining cells were incubated further with 0.25 mg/mL MTT for 3 h. MTT was extracted with 0.04 mol/L HCl/isopropanol, and the color change in the extract was measured at 595 nm. Cell viability was expressed as percentage of OD_{595} to the control group.

Determination of Ornithine Decarboxylase (ODC) Activities in the Gastric Mucosa and MKN-28 Cells

ODC activity was assessed by measuring the amount of $^{14}CO_2$ liberated from DL-[1-^{14}C] ornithine (21). Mucosae were homogenized in 67 mmol/L phosphate buffer (pH 7.4) containing 0.02% Brij-35, 0.5 mmol/L NaF, 10 mmol/L EDTA 0.1 mmol/L pyridoxal phosphate, and 2 mmol/L dithiothreitol for 20 sec and then sonicated for 15 sec under ice-cold conditions. The homogenates were centrifuged at 20, 000 g at 4°C for 20 min. Adherent cells were scraped from the culture plate and placed in 10 mmol/L Tris-HCl buffer (pH 7.4) containing 1 mmol/L EDTA, 0.05 mmol/L pyridoxal phosphate, and 5 mmol/L dithiothreitol. They were sonicated for 15 min under an ice-cold condition and centrifuged for 2,500 g at 4°C for 10 min. A 300 μL aliquot of the supernatant was incubated in a stoppered test tube in the presence of 2.5 mmol/L of L-[1-^{14}C]ornithine for 15 min at 37°C. The $^{14}CO_2$ liberated from the decarboxylation of ornithine was trapped by a piece of filter paper impregnated with 20 μL of 2.0 mol/L NaOH. The paper was placed in a well connected to the stopper and suspended above the reaction mixture. The reaction was terminated by addition of 0.3 mL of 10% trichloroacetic acid. The radioactivity of $^{14}CO_2$ trapped in the filter paper was measured by a liquid scintillation counter (Beckman Instruments). Protein content of the supernatant was measured by the method of Lowry. The enzyme activity was expressed as picomoles $^{14}CO_2$ liberated per milligram of protein per hour.

Assessment of Mucosal Mucus Content

Sections were stained with the Periodic Acid-Schiff (PAS) technique and counterstained with Mayer's hematoxylin. The amount of mucus within the mucosa was assessed by measuring the relative thickness of the mucus-secreting layer (4), using an image analyzer (Q500IW, Leica Imaging Systems Ltd., Cambridge, UK) at a 200x magnification by a person who was unaware of the type of treatment. This method was based on the determination of the length of the gastric pit and isthmus (x) over the total mucosal thickness (y), and finally expressed as a ratio of x/y. Ten fields of measurements were taken per section and averaged.

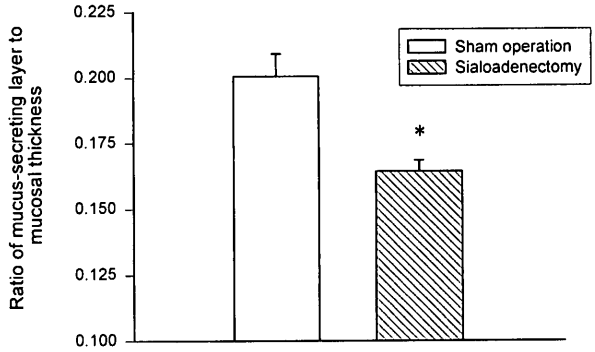

Fig. 1. Effect of sialoadenectomy on basal mucus synthesis in rats. Columns and vertical bars represent means±SEM of 7-8 rats. *P<0.05 vs Sham operation.

Statistical Analysis

Results were expressed as means±SEM. Statistical analysis was performed with an analysis of variance (ANOVA) and the unpaired Student's t-test. P values less than 0.05 were considered statistically significant.

Results

Effect of Sialoadenectomy on Basal Mucus Synthesis

EGF is known to pose protective effect on the gastric mucosa. This growth factor is constantly produced from salivary gland in both rodents and humans. In the present study, removal of the salivary gland (submaxillary glands) from the rats significantly reduced the basal mucus synthesis in the gastric mucosa (Fig. 1).

Effect of Cigarette Smoke Exposure on Mucus Synthesis in Rats and Its Modification by EGF

In our previous study, cigarette smoke exposure (4%) for 3 and 6 days was shown to delay ulcer healing in rats (16). We next measured the effect of cigarette smoke exposure using the same concentration on mucus secretion in the gastric mucosa of intact rats. The ratio of mucus-secreting layer to mucosal

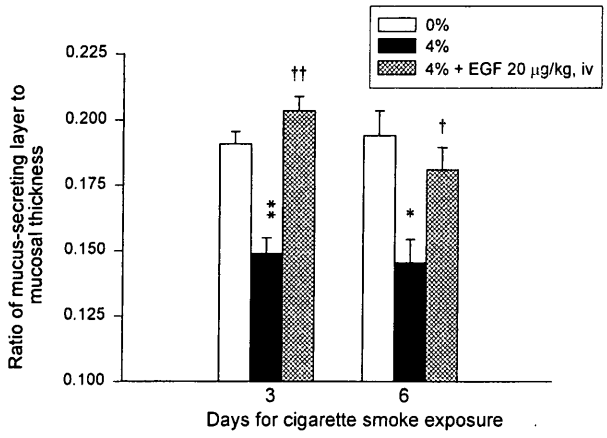

Fig. 2. Effect of epidermal growth factor on mucus synthesis in rats with cigarette smoke exposure. Columns and vertical bars represent means±SEM of 7-8 rats. *P<0.01, **P<0.001 vs corresponding 0%; †P<0.05, ††P<0.001 vs corresponding 4%.

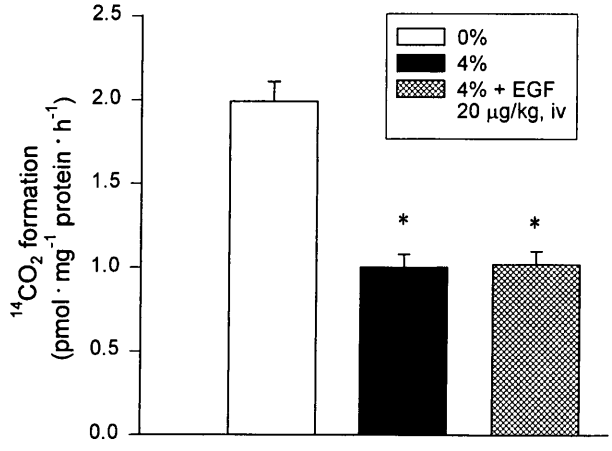

Fig. 3. Effect of epidermal growth factor on gastric mucosal ornithine decarboxylase activity in rats exposed to cigarette smoke for 3 days. Columns and vertical bars represent means±SEM of 7-8 rats. *P<0.001 vs corresponding 0%.

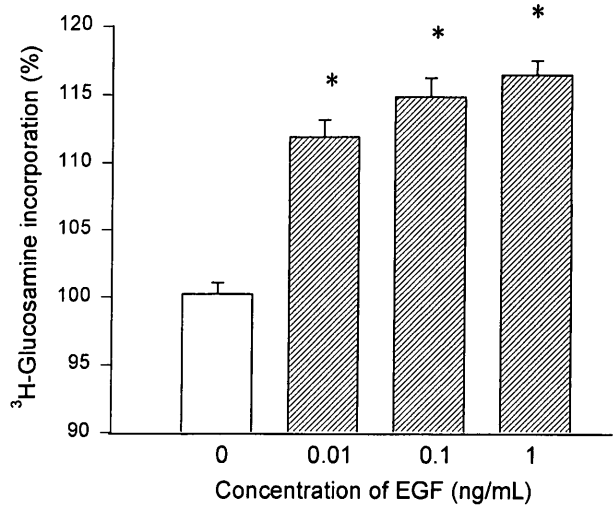

Fig. 4. Effect of epidermal growth factor on mucus synthesis in MKN-28 cells. Columns and vertical bars represent means±SEM of 6 samples. *P<0.001 vs 0 ng/mL.

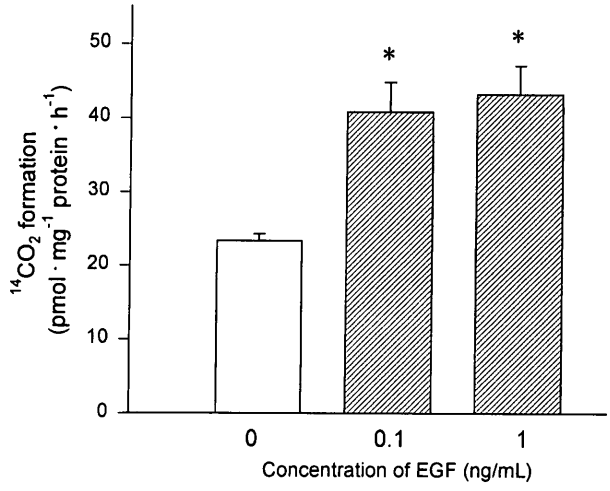

Fig. 5. Effect of epidermal growth factor on ornithine decarboxylase activity in MKN-28 cells. Columns and vertical bars represent means±SEM of 6 samples. *P<0.05 vs 0 ng/mL.

thickness was markedly downregulated when compared to the basal thickness in both 3 and 6 days after cigarette smoke exposure. This reduction, however, was completely reversed by intravenous administration of EGF (Fig. 2).

Effect of Cigarette Smoke Exposure on Gastric Mucosal ODC Activity in Rats and Its Modification by EGF

ODC is a rate-determining enzyme in polyamine biosynthesis. Cigarette smoke exposure for 3 days significantly suppressed the ODC activity in the gastric mucosa (Fig. 3). We evaluated further the relationship between EGF and ODC activity in vivo. Unexpectedly, EGF administration at the dose that could reverse mucus synthesis in smoking rats did not affect the ODC activity in the gastric mucosa.

Effect of EGF on Mucus Synthesis and ODC Activity in MKN-28 Cells

We demonstrated further the effect of EGF on mucus synthesis in MKN-28 cells (a secretory cell line). EGF incubation (0.01-1.00 ng/mL) significantly increased mucus synthesis in MKN-28 cells in a dose-dependent manner (Fig. 4). The incorporations of ^{3}H-glucosamine were significantly higher in the EGF-treated groups when compared to the control (Fig. 4). Furthermore, EGF treatment (0.1 and 1.0 ng/mL) also significantly increased the ODC activity in MKN-28 cells by more than 50% (Fig. 5).

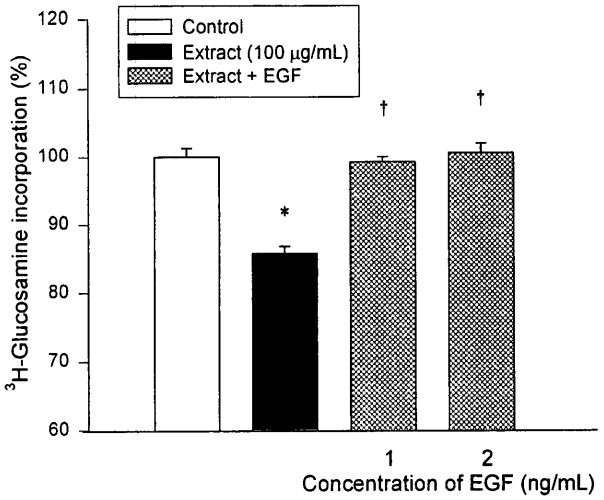

Fig. 6. Effect of epidermal growth factor on mucus synthesis in cigarette smoke extract treated MKN-28 cells. Columns and vertical bars represent means±SEM of 6 samples. *P<0.001 vs Control; †P< 0.001 vs Extract.

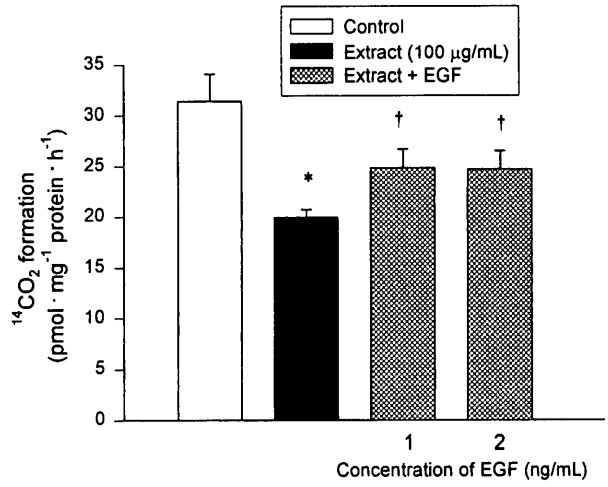

Fig. 7. Effect of epidermal growth factor on ornithine decarboxylase activity in cigarette smoke extract treated MKN-28 cells. Columns and vertical bars represent means±SEM of 6 samples. *P <0.01 vs Control; †P<0.05 vs Extract.

Effects of Smoke Extract on Mucus Synthesis in MKN-28 Cells and Its Modification by EGF

We determined the effect of cigarette smoke extract, which was derived from the chloroform fraction, on mucus synthesis in MKN-28 cells. This fraction was shown previously to potentiate ethanol-induced gastric ulceration in rats (7). Indeed, incubation with this extract (100 μg/mL) significantly suppressed mucus synthesis in the cells (Fig. 6). As expected, EGF treatment (1 and 2 ng/mL) significantly and completely abolished the inhibitory effect of cigarette smoke extract on mucus synthesis.

Effect of Cigarette Smoke Extract on ODC Activity in MKN-28 Cells and Its Modification by EGF

When we assessed the effect of cigarette smoke extract on the ODC activity in the cell system, the enzyme activity was also reduced by smoke extract. EGF, however, unlike the in vivo study, significantly but partially elevated the ODC activity in this cell line (Fig. 7). All agents used in this study did not significantly affect cell viability.

Discussion

Gastric mucus serves the first line of defense of the gastric mucosa and acts as protection against the natural endogenous aggressors (acid, pepsin, bile) or exogenous damaging agents (alcohol, NSAIDs) by forming a stable unstirred layer that supports surface neutralization by bicarbonate (3). It also acts as a lubricant against mechanical damage (2). Finally, it provides protection by scavenging oxidants produced in the gastric lumen (8). The effect of cigarette smoking on mucus secretion is controversial. Hollander et al. (10) reported a reduction in the concentration of acid mucosubstances in the gastric epithelium of smokers when compared with non-smokers. Whereas, Rack and Sonnenberg (20) showed that smoking did not affect gastric mucus secretion in the gastric juice.

EGF has been closely related to mucus secretion regulation in the gastric mucosa. Deprivation of salivary EGF by sialoadenectomy caused a 31% or 38% reduction of adherent mucus thickness or mucin content in rats, respectively (22), which is consistent with the present finding (Fig. 1). *In this study, supplementation of EGF nearly completely restored the normal characteristics occurred in the gastric mucosal mucus coat* (22). These results indicate that salivary EGF is critical for gastric mucus synthesis and secretion. Smoking one cigarette every 30 min resulted in a significant reduction in basal-salivary EGF secretion in healthy human subjects (14). Smoking was also associated with reduced salivary secretion of EGF and with an increased prevalence of peptic ulcer in patients (12). Our results showed that EGF supplementation reversed the depressed gastric mucus content by cigarette smoke or its extract both in vivo and in vitro. However, the exact mechanism associated with this action is undefined.

In studying this phenomenon, the association between EGF and ODC activity was investigated. In the event that mucus synthesis was upregulated by EGF, which also activated ODC activity in MKN-28 cells (Figs. 4 and 5). This finding is in accord with

Wang's finding that exposure of intestinal crypt cells to EGF significantly increased ODC activity (27). In fact, immunocytochemical studies on the gastric epithelia indicate that ODC is present in mucous neck cells and is colocalized with mucus (11). All these suggest that EGF together with polyamines could be involved in the physiologic regulation of mucus synthesis in the stomach.

Cigarette smoke extract markedly reduced mucus synthesis and ODC activity in MKN-28 cells (Figs. 6 and 7). Supplementation of EGF completely reversed the inhibition of mucus synthesis but only partially affected ODC activity. Furthermore, in intact animals, exogenous EGF also did not affect the suppressed activity of ODC in the gastric mucosa while the reduced gastric mucus synthesis was completely reversed. These results suggest that even though ODC was involved in the regulation in mucus synthesis, *it might not be the enzyme responsible for the reversal action of EGF on the inhibitory effect of cigarette* smoke or its extract on mucus synthesis. However, one cannot exclude the possibility that higher dose of EGF could affect ODC activity in both gastric mucosa and epithelial cell line under these pathologic conditions.

In conclusion, cigarette smoke exposure adversely affects the gastrointestinal tract partially through the impairment of mucus synthesis, a defensive barrier in the gastric mucosa. EGF, a mitogenic as well as protective mediator in the gastric mucosa is probably responsible for the maintenance of mucus synthesis under normal physiological condition through the activation of its downstream effectors, possibly ODC pathway. Cigarette smoking is likely to suppress the gastric ODC activities both in vivo and in vitro and also inhibited mucus synthesis. EGF reverses the inhibitory effect of cigarette smoke or its extract on mucus synthesis, but the action is unlikely through the ODC pathway. The present study unveils the possible mechanisms by which cigarette smoking adversely affects the gastrointestinal tract, and further our understanding on the future therapeutic strategy on ulcer disorders, especially those with delayed ulcer healing, in which reduction of mucus secretion is indicated.

Acknowledgments

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