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Pathogenesis of nicotine treatment and its withdrawal on stress-induced gastric ulceration in rats

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Abstract

Previous studies showed that cigarette smoking was closely associated with gastric ulceration. People usually smoke under stress conditions, and together, these could induce more gastric damage. In the present study, we aimed to study the effects of nicotine administration and its withdrawal on stress-induced gastric ulceration in rats. Male Sprague–Dawley rats were given nicotine (25 or 50 μg/ml) for 10 days and then withdrawn for 2, 4 or 6 days. They were subjected to cold-restraint stress for 2 h after nicotine treatment or after nicotine withdrawal, and then killed. The results indicated that both nicotine treatment and its withdrawal potentiated stress-induced gastric damage. The mucosal glutathione (GSH) and mucus levels were reduced by stress and decreased further by nicotine. The prostaglandin E_2 concentration remained unchanged. To conclude, the adverse effect of nicotine on stress ulceration was prostaglandin E_2-independent but mediated by the depression of glutathione and mucus levels in the gastric mucosa.

Keywords: Blood flow; Glutathione; Mucus; Nicotine; Prostaglandin E_2; Gastric ulcer

1. Introduction

Epidemiological study has shown that cigarette smoking and peptic ulcer diseases are closely associated (Kato et al., 1992). Smokers were more often found to have peptic ulcer than non-smokers. Moreover, the recurrence rate was also increased (Sontag et al., 1984) in a dose-dependent manner (Korman et al., 1983). There is a clear evidence of smoking behavior being related with stressful events. Smokers usually consume more cigarettes or inhale more intensely when they are anxious. Since both stress and smoking would affect the prevalence of peptic ulcers (Misiewicz, 1988), it is likely that both conditions could concurrently disturb the gastrointestinal tract and produce adverse effect in the organ.

Nicotine, which is the most important component in tobacco smoke, has long been considered to exhibit many of the tobacco effects (Jaffe, 1985). Because of the well-studied pharmacological properties of the alkaloid, it is used in experiments on effects of cigarette smoking. Nicotine has also been used as nicotine gum and transdermal patches to replace cigarette smoking in humans (Silagy et al., 2000), and continuous administration of nicotine potentiates secretagogue-induced gastric damage (Robert, 1972). Long-term oral administration of nicotine has also been found to induce hemorrhagic gastric ulcers (Chowdhury et al., 1990) and aggravate gastric mucosal injury induced by ethanol (Cho et al., 1990; Hui et al., 1991) as well as by stress (Qiu et al., 1992). Therefore, the study of the effects of nicotine on the stomach has significant clinical implications. However, the exact mechanism of how nicotine produces adverse effects on the stomach is still not well defined.

It has been established that glutathione (GSH) is related to several structural and functional processes of cells. The gastric mucosa contains a high concentration of reduced GSH (Body et al., 1979). The production of GSH was found to be under the influence of cigarette smoking (Joshi et al., 1988). Such thiols would influence the physical properties of the mucus since its subunits are joined by disulfide bridges (Allen, 1978). Considerable evidence indicates that both GSH and mucus play a significant role in mucosal protection. Stress has been found to lower the amount of mucus adhering to the gastric mucosa (Green et al., 1981). It

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has been shown that mucus synthesis was inhibited by about 50% when the tobacco smoke condensate was added to cultured rat antral mucosa (Yeomans, 1988). The exact component involved in such an effect remains unknown. It was found that chronic nicotine administration decreases gastric wall mucus content in rats (Wong et al., 1986). Nevertheless, it is likely that both cigarette smoking and stress could concurrently affect the GSH level in the gastric mucosa, and thereby, adversely alter the consistency of the mucus layer on top of the epithelium, which would greatly alter the defense mechanism of the gastric mucosa. Cigarette smoking and stress also disturb the microcirculation of the mucosa, particularly under stress or cigarette smoking conditions, which would adversely affect mucosal integrity (Ma et al., 1999). Furthermore, prostaglandins also play an important role in the maintenance of gastric mucosal integrity (Whittle and Vane, 1981). The fact that smoking reduces gastric prostaglandin synthesis or release (McCready et al., 1985; Quimby et al., 1986) suggests that chronic nicotine treatment would affect the mucosal prostaglandin E_2 level and affect the susceptibility to ulceration in the stomach. More interestingly, effects on stress ulceration after alkaloid withdrawal have not been reported. This would be equally important and needs further study because many signs and symptoms have been attributed to the cessation of cigarette smoking and these are likely to be caused by nicotine withdrawal.

We aimed to study the effects of continuous administration of nicotine and its withdrawal on stress-induced gastric mucosal damage and also aimed to investigate the possible ulcerogenic mechanisms involving the depletion of GSH, mucus and prostaglandin E_2 levels in the gastric mucosa.

2. Materials and methods

2.1. Experimental animals

The following experiments were approved by the Committee of Use of Live Animals for Research and Teaching of the University of Hong Kong. It also followed the ''Principles of laboratory animal care'' approved by NIH publication No. 85–23, revised in 1985. Female Sprague–Dawley rats weighing between 150 and 180 g were used. Five animals were normally kept in a cage (length: 42 cm; width: 25 cm; height: 20 cm) and reared on a balanced pellet diet of laboratory chow (Ralston Purina, Chicago, IL, USA). All animals were given tap water or a solution of nicotine bitartrate (Sigma, St. Louis, MO, USA) (25 or 50 μg/ml) in tap water to drink for 10 days. These concentrations of nicotine were selected because they produced a similar nicotine serum concentration similar to a heavy cigarette smoker (Hui et al., 1991). The animals were housed in an air-conditioned room with a temperature maintained at 22 ± 1 °C and humidity at 70–75%. They were exposed to a daily cycle of 12-h light and 12-h darkness. The animals were starved for 48 h before experimentation but were allowed free access to 8% sucrose in 0.2% NaCl solution (Glavin and Mikhail, 1976). In the case of the nicotine-treated rats, their sucrose/salt drinking solutions contained the same concentrations of nicotine as in the pre-starvation period. Drinking solutions were removed 1 h before stress.

2.2. Induction of gastric damage

After 48 h of starvation, the animals were restrained inside individual close-fitting tubular wire mesh cages and exposed to a room temperature of 4 ± 0.3 °C, which is a standard method widely used to produce stress ulcer in animals (Qiu et al., 1992). The rats were subjected to 2-h cold-restraint stress either immediately after nicotine treatment or 2, 4 or 6 days after nicotine withdrawal. At the end of the experiment, the animals were anesthetized and the stomach was opened along the greater curvature. Lesion size (in mm) was determined by measuring each lesion along its greatest diameter. In the case of petechiae, five such lesions were taken as the equivalent of a 1-mm lesion. The total lesion lengths in each group of rats were averaged and expressed as the lesion index. After the measurement of lesions, part of the mucosa was cut and fixed in 10% buffered formalin solution. The rest of the glandular mucosa was scraped with a glass slide and immediately frozen in liquid nitrogen before storage at −70 °C until determination of GSH and prostaglandin E_2.

2.3. Measurement of mucus thickness

After fixation in buffered formalin for 24 h, 6-μm sections were made and stained with periodic acid followed by Schiff's reagent. Finally, they were counter-stained with Harris hematoxylin and mounted in Permount. The thickness of the mucus was measured at intervals of 2 mm along the length of each longitudinal strip using a calibrated graticule in the eyepiece. The values were then averaged.

2.4. Measurement of mucosal glutathione (GSH)

The measurement of gastric mucosal GSH level, a non-protein sulfhydryl, followed the procedures developed by Cho et al. (1991). Mucosal tissues were weighed and homogenized in a sodium phosphate buffer solution and centrifuged at 5000 rpm and 4 °C. The deproteinized supernatant was mixed with the sodium phosphate-buffered solution and 5,5′ dithiobis-(2-nitrobenzoic acid). The final yellow color was read against the reagent blank at 412 nm using a spectrophotometer (DU650, Beckman Instrument, Fullerton, CA, USA).

2.5. Measurement of mucosal prostaglandin E_2 level

The mucosal tissues were homogenized in Kreb's solution with indomethacin and centrifuged at 10,000 rpm at 4

Table 1
Effects of chronic (10-day) nicotine treatment and its withdrawal for 2, 4 or 6 days on the mucosal non-protein sulfhydryl (glutathione) (nmol/mg protein) level in the rat stomach

	Treatment period	Withdrawal period		
	Day 10	Day 2	Day 4	Day 6
A. No stress (unrestrained at 22 C) for 2 h				
Control (tap water)	50.43 ± 6.67	49.79 ± 4.95	50.87 ± 4.42	50.65 ± 4.79
Nicotine (25 μg/ml)	47.05 ± 8.37	52.41 ± 8.09	51.39 ± 3.89	49.30 ± 5.77
Nicotine (50 μg/ml)	49.60 ± 6.03	50.82 ± 8.57	51.93 ± 4.92	48.18 ± 3.88
B. Stress (restrained at 4 C) for 2 h				
Control (tap water)	21.86 ± 1.74[a]	20.91 ± 0.86[a]	22.40 ± 0.97[a]	21.72 ± 0.74[a]
Nicotine (25 μg/ml)	16.58 ± 0.96[b,c]	15.75 ± 0.69[a,d]	19.96 ± 1.49[a]	22.65 ± 0.95[a]
Nicotine (50 μg/ml)	15.25 ± 1.04[e,d]	15.73 ± 0.29[c,e]	22.98 ± 2.11[a]	23.53 ± 1.18[a]

Values are means ± S.E.M. for eight rats in each group. All rats were starved 48 h before the start of the experiments.
[a] $P < 0.001$ when compared to its corresponding controls in A.
[b] $P < 0.05$ when compared to the tap water control group in B.
[c] $P < 0.01$ when compared to its corresponding controls in A.
[d] $P < 0.01$ when compared to the tap water control group in B.
[e] $P < 0.001$ when compared to the tap water control group in B.

°C. The supernatant was mixed with methyl oximation reagent. The prostaglandin E_2 level was measured with a prostaglandin E_2[^{125}I] assay kit (Amersham, Arlington Heights, IL, USA).

2.6. Measurement of mucosal protein concentration

The gastric mucosal protein concentration was determined by the method of Lowry et al. (1951). The working solution was mixed with sodium carbonate, sodium hydroxide and sodium tartrate, and finally, hydrated with copper(II) sulfate solution. The reaction was terminated by adding a phenol reagent, and the absorbance was measured with a spectrophotometer at 660 nm against its reagent blank.

2.7. Statistical analysis

The results were expressed as means ± S.E.M. The number of animals was at least six to eight in each group. Differences between the means were analyzed with Student's paired and unpaired *t*-tests and also by the one-way analysis of variance (ANOVA) when appropriate. *P* values of < 0.05 were considered statistically significant.

3. Results

3.1. Effects of nicotine treatment and its withdrawal on stress-induced gastric mucosal damage

Nicotine treatment for 10 days did not produce any significant damage in the gastric mucosa as compared with the tap water control. Cold-restraint stress for 2 h produced hemorrhagic lesions in the glandular mucosa (4.90 ± 0.66 mm). Pretreatment with the concentration of 25 or 50 μg/ml nicotine for 10 days dependently increased the damage to 7.88 ± 0.69 mm ($P < 0.05$) or 11.34 ± 1.53 mm ($P < 0.01$), respectively. Withdrawal of nicotine treatment for 2 days also potentiated stress ulceration to a higher level (9.90 ± 1.39 and 13.13 ± 0.95 mm for

Table 2
Effects of chronic (10-day) nicotine treatment and its withdrawal for 2, 4 or 6 days on the mucosal prostaglandin E_2 level (pg/mg protein) in the rat stomach

	Treatment period	Withdrawal period		
	Day 10	Day 2	Day 4	Day 6
A. No stress (unrestrained at 22 C) for 2 h				
Control (tap water)	651.44 ± 78.99	626.08 ± 59.33	617.42 ± 51.89	623.51 ± 59.04
Nicotine (25 μg/ml)	638.42 ± 50.44	607.67 ± 44.81	656.24 ± 62.96	632.83 ± 63.91
Nicotine (50 μg/ml)	659.04 ± 58.81	612.12 ± 70.09	604.13 ± 40.65	600.17 ± 66.76
B. Stress (restrained at 4 C) for 2 h				
Control (tap water)	434.63 ± 41.09[a]	437.75 ± 53.58[a]	455.10 ± 47.52[a]	437.20 ± 41.89[a]
Nicotine (25 μg/ml)	393.90 ± 55.86[b]	441.33 ± 57.87[a]	364.67 ± 35.24[b]	444.51 ± 42.70[a]
Nicotine (50 μg/ml)	400.02 ± 54.03[b]	387.83 ± 49.61[a]	424.80 ± 52.43[a]	417.82 ± 30.58[a]

Values are means ± S.E.M. for eight rats in each group. All rats were starved 48 h before the start of the experiments.
[a] $P < 0.05$ when compared to its corresponding controls in A.
[b] $P < 0.01$ when compared to its corresponding controls in A.

Table 3
Effects of chronic (10-day) nicotine treatment and its withdrawal for 2, 4 or 6 days on the gastric mucosal layer thickness (mm) after 2-h cold-restraint stress in rats

	Treatment period	Withdrawal period		
	Day 10	Day 2	Day 4	Day 6
Control (tap water)	0.422 ± 0.018	0.422 ± 0.013	0.410 ± 0.009	0.413 ± 0.010
Nicotine (25 μg/ml)	0.393 ± 0.012	0.399 ± 0.011	0.389 ± 0.012	0.403 ± 0.012
Nicotine (50 μg/ml)	0.372 ± 0.013[a]	0.370 ± 0.013[a]	0.382 ± 0.012[a]	0.387 ± 0.010

Values are the means ± S.E.M. for six to eight rats in each group. Mucosal layer thickness was measured at intervals of 2 mm along the length of each longitudinal mucosal strip.
[a] $P < 0.05$ when compared to the tap water control.

the groups of 25 and 50 μg/ml of nicotine, respectively). This adverse effect was sustained to the end of the fourth day after nicotine withdrawal. However, this abstinence effect wore off by the sixth day of nicotine withdrawal. The difference between the lesion index for the nicotine- and the water-pretreated groups was non-significant (water control: 4.83 ± 0.68 mm, 25 μg/ml nicotine: 5.01 ± 0.57 mm, 50 μg/ml nicotine: 6.40 ± 1.29 mm).

3.2. Effects of nicotine treatment and its withdrawal on gastric mucosal glutathione (GSH) level

Table 1 summarizes the influence of nicotine treatment and its withdrawal on gastric mucosal GSH levels in rats under both stress and non-stress conditions. Chronic nicotine treatment for 10 days itself had no significant effect on GSH levels under non-stress condition. Similarly, withdrawal of nicotine for 2, 4 or 6 days made no difference in the GSH level when compared to that of the control animals. However, a significant reduction in the GSH level was observed in the stressed animals as compared to that in their corresponding non-stressed control. Nicotine treatment further augmented this effect, with both doses of nicotine showing a significant difference when compared to the stressed tap water control group. These effects were sustained for another 2 days after withdrawal of the alkaloid and returned to the normal level 4 days after withdrawal of nicotine.

3.3. Effects of nicotine treatment and its withdrawal on gastric mucosal prostaglandin E_2 level

Table 2 shows the influence of chronic nicotine treatment and its withdrawal on gastric mucosal prostaglandin E_2 levels in rats. Prostaglandin E_2 levels in rat stomachs were significantly reduced after cold-restraint stress for 2 h in the control groups. Chronic nicotine treatment or its withdrawal did not significantly affect the prostaglandin E_2 levels under either stressed or non-stressed conditions.

3.4. Effect of nicotine treatment and its withdrawal on mucosal mucus layer thickness

The effect of nicotine treatment and its withdrawal on mucosal mucus thickness after cold-restraint stress is shown in Table 3. The mucus layer thickness was significantly decreased in the group receiving 50 μg/ml nicotine in drinking water. This mucus layer-depleting effect was sustained for up to 4 days after nicotine withdrawal. However, it gradually returned to normal 6 days after the alkaloid treatment was stopped and was comparable with that in the tap water control group.

4. Discussion

The present study not only confirmed fact that chronic nicotine treatment potentiated stress-induced gastric mucosal damage (Qiu et al., 1992) but also demonstrated, for the first time, the effects of abstinence from nicotine on stress ulceration. Indeed, the cessation of smoking typically induces anxiety, irritability or impatience (Hatsukami et al., 1985), which may produce additional stress and contributes to peptic ulceration. The present study demonstrated that nicotine withdrawal for 2 or 4 days intensified stress ulceration in the stomach. The adverse effect on the stomach was even more prominent on the second day after withdrawal than during nicotine treatment. This withdrawal effect on gastric ulceration should have significant clinical implications when the chronic effects of nicotine on the gastrointestinal tract are under study. We therefore studied further the effects of both nicotine treatment and its withdrawal on the stomach and attempted to clarify the mechanisms of their potentiating effects on stress ulceration in rats.

Gastric mucus is found in the stomach as a water-insoluble gel adhering to the mucosal surface and as a viscous mobile solution in the gastric lumen (Allen et al., 1986). The former serves as a first line of defense for the gastric mucosa and protects the stomach from damage by endogenous aggressors such as acid and pepsin or exogenous agents such as alcohol. It forms a stable unstirred layer that allows acid neutralization by bicarbonate produced from mucus neck cells (Allen et al., 1986). It has been reported that acute nicotine administration protects against ethanol-induced gastric mucosal damage in rats, and this is accompanied by an increase in both gastric adherent (Kaunitz et al., 1993) and luminal mucus secretion (Endoh et al., 1991). On the other hand, chronic nicotine treatment leads to gastric mucus depletion (Kaunitz et al., 1993) and a decrease in mucosal thickness (Jarvis and White-

head, 1980). In cultured rat antral mucosa, 50% inhibition of gastric mucus synthesis was observed when tobacco smoke condensate was added into the culture medium (Yeomans, 1988). In the present study, the 10-day nicotine treatment dose dependently decreased the intramucosal mucus layer in stressed rats, suggesting that nicotine in cigarette smoke could decrease the synthesis of mucus in the mucosa and adversely affect the defensive mechanism of the stomach.

Gastric mucus also provides protection by scavenging any oxidants produced in the gastric lumen (Hiraishi et al., 1993). The protective action of GSH in the gastric mucosa can also be explained by the scavenger activity by which it could bind to free radicals and reduce damage to structural proteins (Kosower and Kosower, 1978). This is caused partly by the oxidation of essential sulfhydryl groups through which GSH is consumed (Cho et al., 1991). Excessive production of oxygen free radicals would deplete GSH, and thereby, produce gastric ulceration. In fact, gastric GSH was found to be decreased by cold-restraint stress (Body et al., 1979) or starvation (Strubelt and Hoppenkamps, 1983), both of which produce mucosal damage. In addition, kinetic analysis revealed that the decrease in GSH levels was also due to inhibition of its de novo synthesis (Hirota et al., 1989). In vivo study had indeed shown that the production of GSH was under the influence of cigarette smoking (Joshi et al., 1988). In the present study, chronic nicotine treatment as well as its withdrawal had no influence on the mucosal GSH level under non-stressed conditions. However, in case of 2-h stress, chronic nicotine treatment further augmented the reduction in the GSH level already decreased by stress. This effect was still observed 2 days after withdrawal of the alkaloid. Furthermore, it has been found that when the gastric sulfhydryl content is reduced, the mucus becomes more water-soluble (Allen, 1978). An increased ratio of soluble to insoluble mucus could lead to a higher incidence of ulcer formation (LaMont et al., 1983). This implies that chronic nicotine treatment would indirectly affect the ratio of water soluble to insoluble mucus, and thereby, weaken the protective layer, thus contributing to the ulcer-aggravating effect of the alkaloid.

Although there are data suggesting that the cytoprotective effect of prostaglandin might be mediated through endogenous sulfhydryl (Pihan et al., 1986), it seems unlikely that all the protections provided by endogenous and exogenous agents are mediated by sulfhydryl. Histological and biochemical data indicate that dmPGE_2 attenuates both the extent and depth of glandular mucosal injury, which were independent of the GSH levels in the gastric epithelium (Victor et al., 1989). It has been shown that smoking transiently depresses prostaglandin synthesis in the antrum and the fundus (McCready et al., 1985; Quimby et al., 1986). On the contrary, no alteration in the concentration of prostaglandin E_2 was found in the gastric juice (Fedi et al., 1990). Furthermore, nicotine has no direct effect on the gastric mucosal prostaglandin generation in either in vitro or in vivo preparations (Aaron et al., 1986). In the present study, there was no significant change in gastric mucosal prostaglandin E_2 after chronic nicotine treatment as well as after withdrawal of the alkaloid, suggesting that the ulcer-worsening effect of chronic nicotine treatment and its abstinence was not mediated through prostaglandin depletion.

To summarize, the chronic nicotine treatment dose dependently decreased mucus thickness in stressed animals. Nicotine withdrawal produced the same effect, at least for 4 days. The alkaloid itself had no influence on the GSH and prostaglandin E_2 levels under non-stressed conditions. However, 2-h cold-restraint stress decreased both GSH and prostaglandin E_2 levels in the gastric mucosa. The 10-day nicotine administration further augmented the reduction in GSH but not that of prostaglandin E_2 levels under stress conditions. With an action on GSH, it is likely that nicotine could indirectly affect the proportion of soluble to insoluble mucus, and thereby, weaken the mucosal barrier. Moreover, the reduction in the mucosal GSH level would imply a decrease in the free radical scavenger activity, which could also lead to more ulcer formations in the stomach. Similar ulcerogenic mechanisms also apply to the potentiating effect of nicotine withdrawal on stress-induced gastric ulceration.

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