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• BRIEF REPORTS •

Effects of nitric oxide on gastric ulceration induced by nicotine and cold-restraint stress

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Abstract

AIM: Stress induces gastric ulceration in human and experimental animals. People tend to smoke more cigarettes when under stress. Nitric oxide (NO) and nicotine have opposing effects on gastric integrity. The present study examined the possible therapeutic benefit of NO in nicotine-treated rats with stress-induced gastric ulceration.

METHODS: Rats drank a nicotine solution while control rats drank tap water for 20 days. The alkoloid was then replaced by water with or without supplementation of isosorbide dinitrate (NO donor) for an additional 10 days. Isosorbide dinitrate was given twice shortly before experiments (acute) or three times daily by oral gavages for 10 days after the rats stopped drinking nicotine solution. At the end of experiments, ulcer index, gastric adhesion mucus content and MPO activity were measured and analysed.

RESULTS: Nicotine treatment decreased gastric mucus content and intensified stress-induced gastric ulcer. A higher ulcer index persisted even after the rats stopped drinking nicotine solution for 10 days. Acute NO donor showed no benefit on both mucus and ulcer index in nicotine treatment or/and stress condition. Chronic NO donor treatment reversed the worsening action of nicotine in stomach. Stress increased gastric mucosal myeloperoxidase (MPO) activity, which was antagonized by chronic NO treatment. However, nicotine was unlikely to change mucosal MPO activity.

CONCLUSION: The intensifying action of nicotine on stress-induced gastric ulceration persists for 10 days after cessation. Nicotine treatment significantly decreases gastric mucus content that can be restored by chronic NO donor treatment. The present study suggests that NO antagonizes the ulcerogenic action of nicotine through a cytoprotective way.

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INTRODUCTION

Stress is well associated with cigarette smoking. It has been reported that cigarettes help alleviate the feelings of stress which may underlie the tendency to smoke more cigarettes when under stress. However, smoking also further enhances stress levels because stress level has been shown to decline after smoking cessation[1]. Hence, the vicious cycle of stress and smoking perpetuates. Clinical study and experiments have demonstrated that stress is a risk factor for gastrointestinal tract diseases[2-5] and peptic ulcer disease is more common in smokers than in non-smokers. Although Western and traditional Chinese medicine[6] have demonstrated efficacy in the treatment of peptic ulcer disease, peptic ulcers in smokers heal slowly and relapse frequently[7].

Since nicotine accumulates in gastric juice, nicotine is thought to be the major culprit in cigarette smoking causing the detrimental effects of smoking in gastric ulceration[7]. Nicotine also stimulates the central nervous system and chronic nicotine intake intensifies stress-induced gastric mucosal damages in rats[8,9]. The interaction between stress and nicotine has been reported to work synergistically to intensify gastric ulceration[10,11]. Smoking cessation could reduce the incidence and recurrence rate of peptic ulcer but only in people who were light smokers. However, in heavy smokers, smoking cessation did not reduce the incidence and recurrence rate of peptic ulcer. To treat peptic ulcer disease in these former smokers, more studies are needed to provide insights in to the restoration of the normal gastric integrity and the prevention of disease recurrence. Since gastric mucosal prostaglandin, nitric oxide (NO) and gastric adhesion mucus content[12] could contribute significantly to maintaining normal gastric integrity, *i.e.,* cytoprotection, while neutrophil infiltration had an ulcerogenic effect[13], these important parameters were, therefore, the focus of the present study.

In support of the role of prostaglandin, aspirin which inhibits prostaglandin synthesis has been shown to induce gastric mucosal damage. However, in the presence of an NO releasing compound, the ulcerogenic action of aspirin is abolished[14]. In animal experiments, chronic nicotine treatment has been shown to markedly reduce gastric mucus content[15], while other studies demonstrated that NO enhanced gastric mucus production[16,17]. Inhibition of NO synthesis intensifies, while L-arginine (an NO substrate) reduces gastric ulceration in rats induced by cold-restraint stress[18]. These studies suggest that NO may provide a therapeutic benefit to reverse the detrimental effects of nicotine, especially in heavy smokers. In this study, we used the cold-restraint stress-induced gastric ulceration model to evaluate the role of NO in restoring normal gastric integrity after chronic nicotine insult.

MATERIALS AND METHODS

General

In present study, we used male Sprague-Dawley rats (weighing 155-180 g). The animals were fed with a conventional pellet diet. Three rats were housed per cage and kept at room temperature (22±1℃) with humidity of 65-70%. The rats were randomly assigned to tap water control group and nicotine group. In the nicotine group, the animals drank a nicotine solution [tap water containing nicotine bitartrate (50 μg/ml)

(Sigma)] for 20 days. NO donor was given to some nicotine-treated rats twice at 1 hour and at 10 min before experimentation. In the second experiment, rats drank the nicotine solution for 20 days and then the solution was replaced by tap water. NO donor was administered to some nicotine-treated rats for an additional 10 days. Solid food was withdrawn 48 hours before the start of experimentation. The animals were kept in fasting cages and allowed to drink a solution containing 8% sucrose in 0.2% NaCl (w/v). In the nicotine-treated group, rats were given the 8% sucrose/0.2% NaCl solution containing the same concentration of nicotine.

NO donor

Isosorbide dinitrite (Sigma) was used as an NO donor[19] and administered at a dose of 10 mg/kg body weight. Isosorbide was freshly prepared. For acute treatment, the rats were given isosorbide by oral gavages twice at 1 hour and at 10 minutes before experimentation. In chronic experiments, NO donor was administered by oral gavages every 5 hours, three times daily for 10 days simultaneously after quitting nicotine.

Cold-restraint stress induced gastric ulcer

Rats were starved for 48 hours, and the drinking solution was removed 1 hour before starting experiments. For cold-restraint stress, the rats were restrained in individual close-fitting tubular wire-mesh cages at 4℃[12] and the no stress control group was kept in starvation cages at 22℃. At the end of 2 hours, all the rats were sacrificed.

Gastric ulcer index

An observer who was unaware of the treatment regimen determined the severity of gastric mucosal lesions. The lesions were examined under an illuminated magnifier (3X). Lesion size (mm) was measured along its greatest length and in the case of patches, five such lesions were considered the equivalent of a 1 mm ulcer. The sum of the lesion lengths in each group of animals was divided by its number and expressed as the mean gastric hemorrhagic lesion index[8].

Gastric adhesion mucus content

Gastric adhesion mucus content was determined by the Alcian blue method[12,20]. The stomaches were removed, opened, and immediately soaked in Alcian blue solution for 2 hours. The Alcian blue dye complex attached to the gastric wall mucus and was separated from gastric wall by vortexing and subsequent extracting with magnesium chloride. The extracted solution was quantitated spectrophotometrically at 598 nm. The mucus content was correlated with the amount of Alcian blue per gram wet weight of glandular tissue.

Gastric mucosal myeloperoxidase activity (MPO)

MPO activity was measured as previously described[21-24]. Firstly, the gastric mucosa was scraped after examination of ulcer index. The scrapings were then homogenized in ice-cold phosphate buffer. Hexadecyltrimethyl ammonium bromide (0.5% HTAB) (Sigma) was added to this phosphate buffer (50 mM, pH 6.0) to release MPO from the primary granules of neutrophils. Homogenates were then centrifuged and the supernatants were aspirated and mixed with *o*-dianisidine hydrogen peroxide reagent (Aldrich Chemical Co.) and absorbance at 460 nm was measured with a spectrophotometer. One unit of MPO activity was defined as that degrading 1 μmol of peroxide per minute at 25℃ per g protein of gastric mucosa.

Statistics

All values in present study indicated mean±SD and were analyzed using the two-tailed Student' s *t* test. Differences between groups exposed to the same experimental conditions were analysed by the χ^2 test. The *P* value less than 5% was considered as statistically significant.

RESULTS

Effects of nicotine and acute NO treatment on stress-induced gastric ulceration

As summarized in Table 1A, only petechiae were found in the stomach of animals in the non-stressed control group and the mean ulcer index were unaffected by tap water, nicotine or acute NO donor treatments. Cold-restraint stress induced haemorrhagic ulcers in the glandular mucosa with a 100% incidence as indicated by the mean ulcer index in all stressed animals, whether given tap water, nicotine with or without NO donor (Table 1B). Treatment with nicotine for 20 days intensified stress-induced gastric ulceration significantly but had no effect on non-stressed group. Acute NO administration twice shortly before cold-restraint stress in nicotine-treated animals failed to reduce the ulcerogenic effect of nicotine (Table 1B).

Interestingly, nicotine treatment alone in the non-stressed group significantly reduced the gastric adhesion mucus content as compared to tap water control group, while acute NO treatment failed to reverse the effect of nicotine (Table 1A). When animals were subjected to stress, the adhesion mucus content was reduced to 50% of that of non-stressed group. Nicotine treatment further reduced the adhesion mucus content in stressed animals and acute NO treatment did not reverse the effects of nicotine (Table 1B).

Table 1 Effects of nicotine and acute nitric oxide treatment on gastric ulceration and adhesion mucus content in rats

Experimental group	No. of rats with lesions	Ulcer index (mm)	Adhesion mucus content (μg/g wet glandular tissue)
A. No stress (unrestrained at 22℃ for 2 hours)			
Tap water	2 (7)	0.04±0.03	382±38
Nicotine alone	3 (8)	0.05±0.03	283±27[a]
Nicotine + NO (acute)	2 (8)	0.06±0.04	276±28[a]
B. Stress (restrained at 4℃ for 2 hours)			
Tap water	8 (8)[c]	6.0±0.7	200±17[b]
Nicotine alone	8 (8)[c]	19.0±1.7[ad]	152±14[ab]
Nicotine+ NO (acute)	7 (7)[c]	17.8±1.7[ad]	143±15[ab]

Values are mean±SD. Number of rats in each group is shown in parentheses. Lesions: Only petechiae in stomach were seen in non-stressed condition. Both petechiae and haemorrhagic ulcer in stomach were observed in stressed condition. Tap water: Rats drank water as control. Nicotine alone: Rats drank nicotine water (50 μg/ml) for 20 days. Nicotine + NO (acute): Rats drank nicotine water (50 μg/ml) for 20 days. Rats were given NO donor 60 min and 10 min twice by oral gavages before stress. [a]*P*<0.05 when compared to its own tap water control in A or B. [b]*P*<0.05, [c]*P*<0.01, [d]*P*<0.001 when compared to the corresponding control in A.

Effects of nicotine and chronic NO treatment on stress-induced gastric ulceration

To mimic the early effects of quitting smoking, animals were given nicotine solution for 20 days and nicotine was replaced by tap water for an additional 10 days and this treatment group was designated as quit nicotine. In the absence of cold-restraint stress, only petechiae but no ulcers were detected in the stomach in all groups: tap water control, quit nicotine cessation with or without chronic NO treatment (Table 2A). Interestingly, the adhesion mucus content was reduced even 10 days after nicotine cessation and chronic NO treatment for 10 days

restored the adherent mucus content to that of tap water control group (Table 2A). Hence, the damaging effect of nicotine on adhesion mucus content persisted even after nicotine cessation but could be reversed by chronic NO treatment.

As shown in Table 2B, cold-restraint stress induced haemorrhagic ulcers in the glandular mucosa in all treatment groups: tap water, quit nicotine with or without chronic NO treatment. The effects of nicotine persisted even after nicotine cessation as indicated by the increased gastric ulcer index in the quit nicotine group as compared to the tap water control group. Consistent with the protective effect of chronic NO treatment in restoring adhesion mucus content in non-stressed animals (Table 2A), the mean ulcer index induced by stress was reduced by NO treatment after nicotine cessation to the level of the tap water control group (Table 2B). In addition, chronic NO treatment after nicotine cessation in stressed animals also restored the mucus content to that of the tap water control group (Table 2B).

Table 2 Changes of ulcer index and adhesion mucus content after quit nicotine intake alone or with chronic nitric oxide donor (NO) treatment

Experimental group	No. of rats with lesions	Ulcer index (mm)	Adhesion mucus content (μg / g wet glandular tissue)
A. No stress (unrestrained at 22℃ for 2 hours)			
Tap water	2 (7)	0.03±0.02	391±39
Quit nicotine	3 (8)	0.04±0.02	278±29[a]
Quit nicotine+NO (chronic)	2 (7)	0.04±0.03	386±37
B. Stress (restrained at 4℃ for 2 hours)			
Tap water	8 (8)[c]	6.2±0.7[d]	190±18[d]
Quit nicotine	8 (8)[c]	9.0±0.9[ad]	142±16[c]
Quit nicotine+ NO (chronic)	7 (7)[b]	5.8±0.8[d]	210±19[d]

Values are mean±SD. Number of rats in each group is shown in parentheses. Lesions: Only petechiae in stomach were seen with non-stressed condition. Both petechiae and haemorrhagic ulcer in stomach were observed in stressed condition. Tap water: Rats drank water as control. Quit nicotine: Rats drank nicotine (50 μg / ml) for 20 days, then, nicotine was replaced by water for another 10 days. Quit nicotine + NO (chronic): Rats drank nicotine (50 μg / ml) for 20 days, then, nicotine was replaced by water, and rats were given NO donor three times daily by oral gavages for 10 days before experiment. [a]$P<0.05$ when compared to its own tap water control in A or B. [b]$P<0.05$, [c]$P<0.01$, [d]$P<0.001$ when compared to the corresponding control in A.

Effects of nicotine and NO on gastric mucosal MPO activity

Since gastric ulcer formation was associated with MPO activity, we next measured gastric MPO activity. As shown in Table 3, the gastric mucosal MPO activity in non-stressed tap water control group was used as a baseline for comparison with other treatment groups. Cold-restraint stress alone induced MPO activity significantly. Although nicotine treatment intensified stress-induced gastric ulceration (Table 1B), the MPO activity was not further increased by nicotine treatment in stressed animals and was slightly but not significantly reduced by acute NO treatment (Table 3). After nicotine cessation, the higher MPO activity was maintained in stressed animals but was significantly suppressed by chronic NO treatment.

DISCUSSION

Gastric ulceration is resulted from an imbalance of gastric defensive and aggressive factors. The adherent mucus layer could provide a defensive barrier against self-digestion by gastric acid and pepsin[25,26]. In a water immersion restraint stress model, gastric ulcer formation was associated with MPO activity[13]. Because MPO is mainly produced by neutrophils, MPO activity is considered as an index for the evaluation of neutrophil infiltration[13]. Since activated neutrophils produce many enzymes and free radicals that damage the gastric mucosa, neutrophil is considered as an aggressive factor in ulcer formation. Chow *et al.*[27] reported that cigarette smoking significantly potentiated the formation of gastric mucosal lesions and enhanced gastric mucosal MPO activity. Reduction of NO production was well associated with enhancement in both neutrophil infiltration and gastric ulcer formation in water immersion restraint stress model[28].

In the cold-restraint stress model, gastric ulcer formation was mainly due to gastric hypermotility, which could lead to mucosal over friction[29]. Hence, the gastric mucus layer is extremely important and the mucus is generally believed to contribute to a cytoprotective action. Gastric mucus originates from the goblet cells and NO plays a critical role in the maintenance of goblet cell functions. NO donors could increase mucus release from gastric mucosal cells in rats[30] and enhance mucus gel thickness in the rat stomach[16]. Tobacco smoking has been reported to suppress NO release[31-33] which might be explained in part by nicotine-induced damage of endothelial cells and lower NO synthesis[34].

In the present study, we examined the effects of nicotine and NO in cold-restraint stress. To mimic the nicotine intake in heavy smokers, animals were given drinking water containing 50 μg/ml of nicotine, a concentration reported to be comparable to the nicotine intake in heavy smokers[8, 35]. This concentration of nicotine in drinking water has been previously shown to intensify cold-restraint stress-induced gastric ulceration[8,9,29]. Our data suggest that chronic nicotine treatment could significantly reduce the gastric mucus content and this latter effect persisted for even 10 days after nicotine cessation. Reduction of the gastric mucus content could lead to weakening of the gastric defensive capability which underlies the intensifying effect of nicotine on stress-induced gastric ulceration. Our results confirmed the previous observations that chronic nicotine administration could reduce the gastric mucus content[36,37].

Table 3 Change in mucosal MPO activity after nicotine intake and nitric oxide donor (NO) treatment

	Tap water +no stress	Tap water +stress	Nicotine +stress	Nicotine +stress +NO (acute)	Quit nicotine +stress	Quit nicotine +stress +NO (chronic)
MPO (u / g protein):	6.3±0.7	9.3±1.1[a]	9.1±1.0[a]	7.9±0.9	9.4±1.2[a]	5.7±0.9[b]

Values are mean±SD. Each group had 8 rats. Stress: restrained at 4℃ for 2 hours. Tap water: Rats drank water (control). Nicotine: Rats drank nicotine 50 μg / ml for 20 days. Quit nicotine: Rats drank nicotine (50 μg / ml) for 20 days, then, nicotine was replaced by water for another 10 days. NO (acute): Rats drank nicotine (50 μg / ml) for 20 days, the rats were then given NO donor 60 and 10 min by oral gavages before stress. NO (chronic): Rats drank nicotine (50 μg / ml) for 20 days, then, nicotine was replaced by water, the rats were given NO donor three times daily by oral gavages for 10 days before experiment. [a]$P<0.05$ *vs* its own corresponding group in tap water + no stress control. [b]$P<0.05$ *vs* its own corresponding group in tap water +stress control.

Although nicotine intensified stress-induced gastric ulceration which was associated with enhanced MPO activity, nicotine had no additive effect on MPO activity, suggesting that the potentiating effect of nicotine is unrelated to gastric mucosal neutrophil infiltration. Acute NO donor treatment had no protective effect on animals subjected to chronic nicotine treatment and stress, while chronic NO donor treatment for 10 days after nicotine cessation could sufficiently restore the normal gastric mucosal integrity and increase the mucus content. Similarly, acute NO donor treatment did not significantly affect MPO activity in animals subjected to nicotine and stress. However, after nicotine cessation, the MPO activity was significantly reduced by chronic NO treatment in stressed animals. These results suggest that prolonged NO therapy may be necessary to restore the normal mucus layer and reduce MPO activity. Nevertheless, our data demonstrate that NO has a gastric mucosal cytoprotective activity and suggest that the protective role of NO in restoring the gastric mucosal integrity may be mediated via suppression of neutrophil infiltration and MPO activity. Our results are of clinical significance, and suggest that prolonged NO therapy may help to restore gastric mucosal integrity in heavy smokers, especially after they have stopped smoking.

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