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Novel Insights on the Effect of Nicotine in a Murine Colitis Model

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ABSTRACT

Studies showed that nicotine has a positive influence on symptoms of ulcerative colitis. In the present study, we explored the effect of nicotine treatment using different routes of administration in the dextran sodium sulfate (DSS) colitis mouse model. We also investigated the effects of cotinine, a major metabolite of nicotine, in the model. C57BL6 adult male mice were given DSS solution freely in the drinking water for seven consecutive days, and tap water was given thereafter. Disease severity, length of the colon, colon tissue histology, and inflammatory markers, including colonic myeloperoxidase activity and colonic tumor necrosis factor-α levels, were evaluated. The effect of nicotine and cotinine treatments via various different routes of administration were examined the DSS model. In addition, we measured the plasma levels of nicotine and cotinine in our treatment protocols. Administration of low, but not high, doses of oral nicotine in DSS-treated mice resulted in a significant decrease in disease severity, histologic damage scores, as well as colonic level of tumor necrosis factor-α. However, the anti-inflammatory effect of nicotine was not seen after chronic s.c. or minipump infusion of the drug. Differences in plasma levels of nicotine and cotinine do not seem to account for this lack of effect. Finally, oral cotinine alone failed to show a significant effect in the DSS model of colitis. These results highlight that dose and route of administration play a critical role in the protective effect of nicotine in the DSS mouse colitis model.

Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease of unknown etiology, which is due, in part, to genetic susceptibility to environmental factors that misdirect the mucosal immune system response to attack the epithelial layer of the colon, causing inflammation. UC is a remitting and relapsing inflammatory condition that results in excessive mononuclear cell and neutrophil infiltration into mucosa and submucosa vicinities that primarily affects the distal colon and rectum (Hanauer, 1996). Patients typically present with bloody diarrhea and passage of pus and/or mucus that often persists for months and is accompanied by abdominal cramping during bowel movements.

UC has been linked to tobacco smoking for more than three decades. Tobacco smoking was often reported to have a beneficial outcome on the course of activity of UC (Boyko et al., 1987; Mokbel et al., 1998; Gheorghe et al., 2004; Höie et al., 2007) and to decrease the need for colon surgery (Odes et al., 2001; Cosnes, 2004). Smokers with UC who quit smoking experienced an increase in disease activity, and symptoms improved in ex-smokers who returned to smoking (de Castella, 1982; Motley et al., 1987; Rudra et al., 1989; Kuisma et al., 2004). Although cigarette smoke contains hundreds of substances, there is evidence that nicotine and/or its metabolites, such as cotinine, account for the beneficial effect of smoking.

A number of clinical studies has been carried out in which nicotine was administered to UC patients in different formulations using patches and gums. Nicotine showed positive results on disease symptomology in some trials. Nevertheless, results are conflicting, and their interpretation has been confounded by side effects experienced by individuals as a result of the high systemic nicotine concentrations required (Pullan et al., 1994; Cosnes, 2004; McGilligan et al., 2007). In addition, it was reported that nicotine lacks efficacy in treating disease relapses and remission (Perera et al., 1984; Thomas et al., 1995; Ingram et al., 2005). Thus, collectively these results indicate that, although transdermal nicotine may be effective for UC, especially in ex-smokers, its use is limited by its adverse event profile. However, it is not clear whether the delivery of nicotine through patches and gum formulations, which do not mimic the intermittent delivery of nicotine from cigarettes, plays a role in the efficacy of the drug. Furthermore, clinical studies conducted by Green et al. (1997) and Sandborn et al. (1997) tested nicotine enemas, in which nicotine is applied directly to the colon; they indicated that UC symptoms, endoscopic features, and histological

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ABBREVIATIONS: ANOVA, analysis of variance; AUC, area under the curve; DAI, disease activity index; DSS, dextran sodium sulfate; HPLC, high-performance liquid chromatography; ISTD, internal standard; MPO, myeloperoxidase; MS/MS, tandem mass spectrometry; TNF, tumor necrosis factor; UC, ulcerative colitis.

damage were improved, with only a few of the patients reporting some side effects. These initial studies indicate that nicotine applied directly to the colon, perhaps in an enema formulation, may be beneficial while causing fewer side effects.

Similar findings with nicotine were reported in rodent models of colitis. For example, oral nicotine treatment improved the macroscopic damage of experimental colitis (Eliakim et al., 2001). Ghia et al. (2006) reported that 20 μg/ml nicotine in drinking water decreased clinical signs of inflammation and reduced colonic myeloperoxidase (MPO) activity and tumor necrosis factor (TNF)-α levels in the mouse dextran sodium sulfate (DSS) model. However, chronic i.p. injection of nicotine (0.25 and 2.50 μmol/kg) in DSS-induced colitis in mice failed to decrease clinical signs of inflammation, colonic TNF levels, and histologic features of the disease (Snoek et al., 2010).

In the present study, we used the DSS mouse model, one of the best described and widely used animal models of UC, because it shows some resemblance to human UC in both clinical (body weight loss, loose stool, bloody diarrhea or rectal irritation, and hematochezia) and histopathologic findings (Okayasu et al., 1990; Cooper et al., 1993; Gaudio et al., 1999). Furthermore, the model is characterized by an excessive T helper cell response (Targan and Karp, 2005). The responsiveness of the DSS-induced colitis mouse model to conventional therapeutic agents for human UC supports its suitability as a model of human UC (Björck et al., 1997). We examined the effect of chronic nicotine administration using various doses and routes of administration (subcutaneous, infusion using osmotic minipump, and oral) to define the optimal route and dose that resulted in ablation of DSS-induced colitis in the mouse. We also tested the effect of one of the main nicotine metabolites, cotinine, to garner insight as to whether it contributes to the beneficial effect of cigarette smoking in UC patients.

Materials and Methods

Animals

Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were 8–10 weeks of age at the start of the experiments, weighed 25–30 g, and were group-housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care–approved animal care facility with ad libitum access to food and water. Mice were housed under standard conditions for a minimum of 1 week before experimentation. Experiments were performed during the light cycle (between 7:00 a.m. and 7:00 p.m.), and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs

(−)-Nicotine hydrogen tartrate salt [(−)-1-methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt] and (−)-cotinine were purchased from Sigma-Aldrich Inc. (St. Louis, MO). DSS (molecular mass 36,000–50,000 kDa) was obtained from ICN Biomedicals Inc. (Aurora, OH). Doses are expressed as the free base of the drug.

Chronic Nicotine Treatment

DSS-Induced Colitis Model. DSS was added to the drinking water at 1, 2.5, and 5% concentrations (in % w/v) for 7 days. On day 8, mice received drinking water without DSS. Controls were all age- and time-matched and consisted of mice that received regular tap drinking water for the corresponding number of days. Each treatment group consisted of 8–10 mice. The following studies were conducted.

Study 1. DSS dose response curve. Separate groups of mice were allocated to the following treatments: water only (controls), 1% DSS, 2.5% DSS, and 5% DSS for 7 days.

Study 2. Effect of oral (−)-nicotine administration. Different doses of (−)-nicotine (6, 12.5, 25, 50, and 100 μg/ml) were added to the drinking water 3 days before and for 7 days along with the induction of colitis with DSS 2.5%.

Study 3. Effect of s.c. (−)-nicotine administration. Saline or (−)-nicotine treatment (0.1, 0.5, 2 mg/kg) was given s.c. twice daily for 10 days. (−)-nicotine was dissolved in physiologic saline (0.9% sodium chloride) and administered by s.c. injection at a volume of 10 ml/kg body weight. The s.c. nicotine or saline injection was administered 3 days before induction of DSS colitis and was continued during the DSS treatment period.

Study 4. Effect of (−)-nicotine administration through minipump. Nicotine was infused for 14 days through an osmotic minipump at a dose of 2.5 or 25 mg/kg per day. Mice were implanted s.c. with Alzet osmotic minipumps (model 2002 [14 days], Durect Corporation, Cupertino, CA), filled with (−)-nicotine or saline solution, under sterile conditions with sodium pentobarbital anesthesia (45 mg/ml i.p.). An incision was made in the back of the animal, and a pump was inserted. The wound was closed with wound clips, and the animal was allowed to recover before being returned to its home cage. The concentration of nicotine was adjusted according to animal weight and the minipump flow rate, resulting in 2.5 or 25 mg/kg per day for 14 days. DSS was added to the drinking water for 7 days in the second week after saline or (−)-nicotine minipump implantation.

Study 5. Effect of oral cotinine administration. (−)-Cotinine (25 or 250 μg/ml) was added to the drinking water 3 days before and for 7 days along with the induction of colitis with DSS 2.5%.

Assessment of the Severity of Colitis: Disease Activity Index

Disease Activity Index (DAI) is the combined score of four clinical parameters: weight loss, stool consistency, rectal irritation, and blood in the stool. Scores were defined as follows: for weight: 0, no loss; 1, 5–10% weight loss; 2, 10–15% weight loss; 3, 15–20% weight loss; and 4, 20% weight loss; for irritation around the anal area; 0, normal; 1, mild irritation; 2, moderate irritation; and 3, severe irritation; for stool consistency: 0, normal; 1, mild loose stool; 2, moderate loose stool; and 3, diarrhea; and for bleeding: 0, no blood; 1, presence of blood (Hemoccult II positive; Beckman Coulter, Fullerton, CA); and 2, gross blood. DAI symptoms were recorded in a blinded fashion at the same time every day and were scored from days 0 to 8. Total DAI score ranged from 0 to 12. On day 8, after replacing the DSS with water, mice were sacrificed, the abdominal cavity was opened, and the colon was immediately removed and its length (cm) measured. To assess the change in DAI over time, results were also expressed as mean ± S.E.M. of area under the curve (AUC) from days 1 to 8. For the nicotine s.c., minipump, and oral groups, blood was taken for the measurement of nicotine plasma levels.

Colonic Histology Assessment

Seven days after the beginning of the DSS treatment, mice in each group were sacrificed, and their colons were removed. Formalin-fixed colon segments were paraffin embedded, and 3-μm sections were stained with H&E. Colonic damage was scored in a blinded fashion based on a published scoring system that considers architectural derangements, epithelium changes, goblet cell depletion, ulceration,

and degree of inflammatory cell infiltrate (Iba et al., 2003). The histologic scoring system was used to quantify the degree of colitis. The total histologic score ranged from 0 to 12, which represented the sum of scores from 0 to 3 for loss of epithelium (0, none; 1, 0–5%; 2, 5–10%; and 3, >10%), for crypt damage (0, none; 1, 0–10%; 2, 10–20%; and 3, >20%), for depletion of goblet cells (0, none; 1, mild; 2, moderate; and 3, severe), and for infiltration of inflammatory cells (0, none; 1, mild; 2, moderate; and 3, severe). The number of infiltrated inflammatory cells in 10 randomly selected power fields (40×) was counted, and the number per 10 fields was calculated. For each analysis, the scores were assigned by one experienced histopathologist who had no knowledge of the group being examined. The histologic colitis score of individual mice is the sum of the different histologic subscores. Light microscope images were acquired with an Axio Scope AX10 microscope and AxioVision 4.6 software (Carl Zeiss Inc., Thornwood, NY). Results are expressed as mean ± S.E.M. with three or four mice per group.

Assessment of Colonic MPO Activity

Colonic tissues were homogenized in phosphate-buffered saline for the assessment of MPO. Samples were homogenized in 100 mM sodium acetate (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide and 5 mM EDTA. The homogenate was sonicated briefly and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was added to a homogenizer solution in a 96-well plate. A total of 75 μl of 3,3′5,5′-tetramethylbenzidine substrate was added to each well, and the plates were incubated for 2 min; 50 μl of stop solution (2 N H_2SO_4) was then added. The plates were read at 450 nm within 30 min. For each mouse, a semilog curve of sample dilution versus optical density was plotted to obtain a midpoint titer. Five to eight animals were used per group.

TNF-α Levels

The colonic sample was homogenized in 1 ml of Tris-HCl buffer containing protease inhibitors (Sigma-Aldrich). Samples were centrifuged at 3000 rpm at a temperature of 4°C for 30 min, and the supernatant was frozen at −80°C until assay. The protein concentration was determined by the Bradford assay (Bradford, 1976). The TNF-α level (pg/mg protein) was determined using a commercial enzyme-linked immunosorbent assay kit (Quantikine M murine; R&D Systems, Minneapolis, MN).

Nicotine and Cotinine Plasma Levels

Blood samples were taken from mice receiving different protocols of nicotine: 10 days after oral (−)-nicotine or (−)-cotinine administration in the drinking water, 2 weeks after continuous minipump (−)-nicotine infusion (2.5 or 25 mg/kg), or 5 minutes after the last s.c. injection of nicotine (0.5 or 2 mg/kg) (Table 1). Animals were anesthetized with CO_2, blood samples were taken by intracardiac puncture just before death, and blood was kept in sodium heparin blood-collection tubes and centrifuged (1400 × *g*, 10 min). The serum was stored at −4°C. Nicotine and cotinine serum levels were measured using high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) analysis. At least six animals were used per group.

Nicotine and Metabolite HPLC/MS/MS Analysis

Specimen Extraction. A total of 50 μl of internal standard (ISTD) containing 50 ng of nicotine-d4 and cotinine-d3 in methanol was added to a 200-μl aliquot of whole blood with mixing. Then, 100 μl of 5 M ammonium hydroxide was added to each sample, followed by 2 ml of methylene chloride. The samples were mixed for 2 min and then centrifuged for 5 minutes at 3000 rpm at 4°C. The organic layer was transferred to a clean test tube. The aqueous phase was extracted twice more with 2 ml of methylene chloride. The organic phases were combined, and 500 μl of 25 mM HCl in methanol was added. Samples were evaporated to dryness under a gentle stream of nitrogen. They were reconstituted with 100 μl of mobile phase and placed in autosample (HPLC/MS/MS) vials for analysis.

Instrumental Analysis. The HPLC/MS/MS system used was an Applied Biosystems 3200 Q TRAP with a turbo V source for TurbolonSpray and a Shimadzu SCL HPLC system controlled by Analyst 1.4.2 software. The chromatographic separation was performed using a Hypersil GOLD column, 3 × 50 mm, 5 μm (Thermo Fisher Scientific, Waltham, MA). The mobile phase contained 10 mM ammonium formate/methanol (10:90 v/v) and was delivered at a flow rate of 0.5 ml/minute. The acquisition mode used was multiple reaction monitoring in a positive mode. Transition ions were monitored for nicotine (163 > 130; 163 > 117), nicotine-d4 (167 > 134), cotinine (177 > 80; 177 > 98), and cotinine-d3 (180 > 80). The total chromatographic separation time for each extract injection was 2 minutes. A calibration curve ranging from 12.5 to 500 ng/ml was constructed for each compound based on linear regression using the peak area ratios of the drug to its deuterated ISTD. Cotinine-d3 was also used as the ISTD for 3-hydroxycotinine.

Statistical Analysis

Statistical analysis of all experiments was performed with analysis of variance (ANOVA). Two-way repeated-measures ANOVAs were used to analyze the data at the different time points. Significant overall ANOVAs were followed by Tukey's post hoc test when appropriate. All differences were considered significant at $P < 0.05$. The GraphPad Prism program was used for data calculations, graphical representations, and statistical analysis (GraphPad Software Inc., San Diego, CA).

Results

Characterization of DSS Dose-Response Curve in the Mouse. We first evaluated colitis severity in the mouse DSS model. For that, we examined the effects of 1, 2.5, and 5% DSS on the DAI scores, colon length, colonic histologic damage, colonic MPO activity, and colonic TNF-α levels in C57BL/6J mice.

TABLE 1

Summary of plasma levels of nicotine and its metabolite cotinine after chronic nicotine treatment and chronic oral cotinine treatment

Results are expressed as mean ± S.E.M. of plasma concentrations in ng/ml.

	Chronic Nicotine Treatment Dose						Chronic Cotinine Treatment Dose	
	Injection (mg/kg)		Infusion (mg/kg/day)		Oral (μg/ml)		Oral (μg/ml)	
	0.5	2	2.5	25	25	100	25	250
Nicotine (ng/ml)	51 ± 4.7	163 ± 12	13 ± 1.5	97 ± 89	18 ± 7.4	27.5 ± 12	—	—
Cotinine (ng/ml)	45 ± 6.5	97 ± 7	23 ± 5	282 ± 210	32 ± 7	240 ± 61	89 ± 33	1336 ± 356

Oral DSS administration for 7 days induced signs of colitis in C57BL/6J mice in a dose-related manner [$F(3,30) = 46.12$, $P < 0.0001$], as measured by the DAI scores (Fig. 1, A–C). Mice treated with 1% DSS did not show significant differences in any of the colitis parameters, including rectal irritation, loose stool, bloody diarrhea, and loss in body weight, compared with the water-treated control group. However, both 2.5 and 5% DSS concentrations significantly increased all clinical signs of colitis. The loss of body weight was significant on day 5, and it gradually increased in mice receiving 2.5% DSS and 5% DSS, with an ~15% weight loss noted on day 8 (Fig. 1C). Similar to DAI, the decrease in colon length was dose related, with all DSS concentrations resulting in a statistically significant reduction in length compared with the water-treated control group (Fig. 1D).

In general, the increase in the inflammatory marker MPO and the proinflammatory cytokine TNF-α correlated with the severity of DAI. MPO activity was undetectable in the water-treated control animals and was higher in mice-treated with 2.5 and 5% DSS [$F(3,14) = 7.668$, $P = 0.0029$] (Fig. 2A). The level of TNF-α, an acute inflammatory marker, was increased significantly in mice treated with 2.5 and 5% DSS compared with the control group [$F(3,16) = 49.46$, $P < 0.0001$] (Fig. 2B).

The histology of the colon after 7 days of DSS treatment was characterized by multifocal changes in crypts, with some areas showing focal lesions, and inflammatory cell infiltration that included neutrophils and lymphocytes. The histologic damage was significantly higher in the 2.5 and 5% DSS groups compared with the control group [$F(3,36) = 36.23$, $P < 0.05$] (Fig. 3E). There were changes suggesting a discontinuous appearance of histologic features with varying severity of epithelial loss (Fig. 3A), shortening of crypts (Fig. 3B), depletion of goblet cells (Fig. 3C), and inflammatory cell infiltration (Fig. 3D). The extent of the inflammatory response also was assessed by histology of mucosal tissue using H&E staining (Fig. 4). DSS-treated mice showed extensive ulceration of the mucosa, with destruction and inflammation mainly in the mucosa; however, some inflammation of the submucosa, as well as shortening of the crypts and inflammatory cell infiltration, was seen with 5% DSS (Fig. 4D). Because the 7-day 2.5% DSS administration protocol induced reliable inflammatory colitis with a low mortality compared with the 5% DSS group (30 versus 75%, respectively, 10 days post-DSS), it was chosen for the subsequent studies with nicotine.

Oral Nicotine Administration Attenuates DSS-Induced Colitis. Oral nicotine treatment at doses of 12.5, 25, and 50 μg/ml significantly decreased DAI scores during the last 3 days of the DSS-induced disease course [$F(3,48) = 6.615$, $P = 0.0021$] (Fig. 5A). In contrast, the highest dose of nicotine (100 μg/ml) significantly enhanced DAI scores at days 7 and 8 compared with the DSS-treated group, as seen in the dose-response curves and AUC values [$F(2,19) = 5.593$, $P = 0.0123$] (Fig. 5, A and B). A similar profile was seen with regard to weight loss; the effect of nicotine was lost at higher doses (Fig. 5C). Nicotine treatment did not significantly reverse the effect of DSS on colon shortening (Fig. 5D).

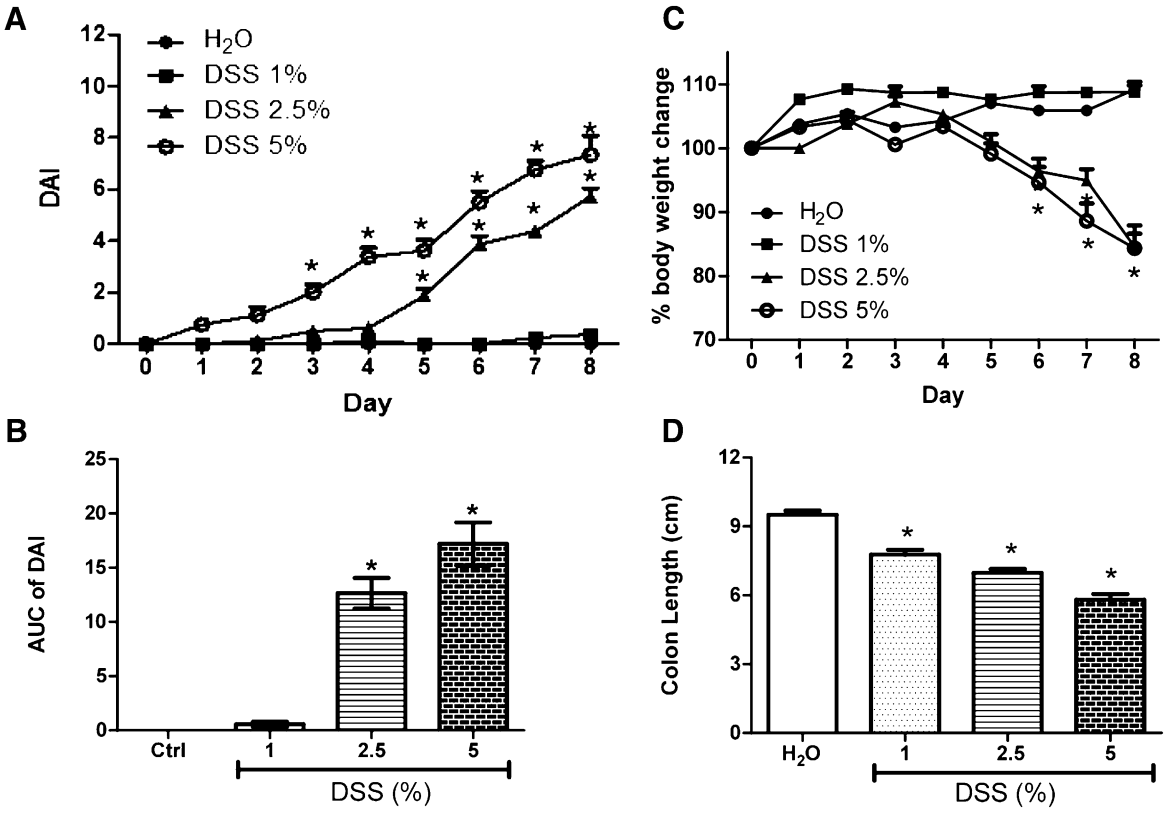

Fig. 1. Colonic inflammation was aggravated after DSS exposure to mice in a dose-related manner. (A) DAI changes among groups exposed to different doses of DSS. (B) AUC of DAI for mice in (A). (C) Percentage of body weight change for mice in (A). (D) Mean colon length (cm) for mice in (A). C57BL/6 mice were given different doses of DSS (1, 2.5, and 5%) in the drinking water for 7 days. All clinical signs were assessed on a daily basis for each mouse and were averaged per day for each group. Results are expressed as mean ± S.E. (n = 6–8). *$P < 0.05$. Ctrl, water-treated animals.

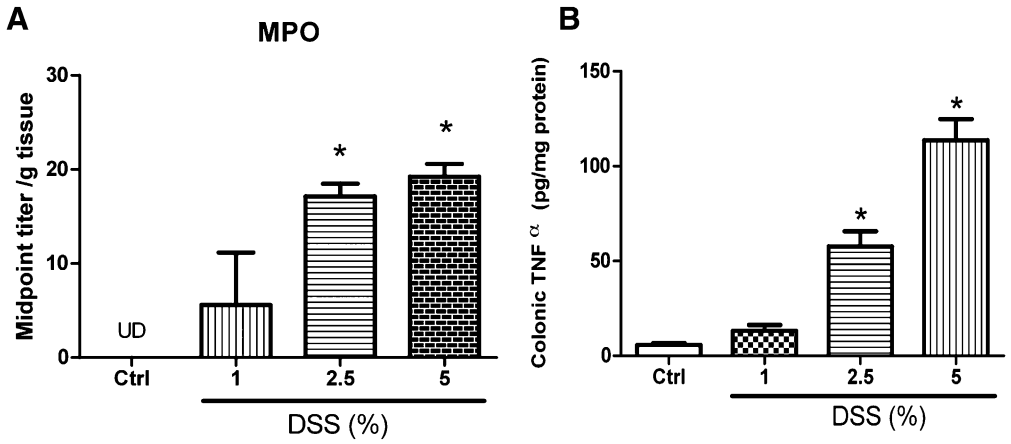

Fig. 2. Aggravation of inflammatory markers after 7 days of exposure to different doses of DSS in mice. (A) Colonic MPO activity (midpoint titer/g wet tissue) in groups exposed to different doses of DSS (0, 1, 2.5, 5%). (B) Colonic TNF-α level (pg/mg protein) for mice in (A). Results are expressed as mean ± S.E. (n = 6–8). *$P < 0.05$. Ctrl, water-treated group.

Oral nicotine at 25 μg/ml, a dose that attenuated DSS-induced colitis signs, also reduced the increase in colonic TNF-α levels seen in DSS-treated mice [$F(3,27) = 4.60$, $P = 0.010$] (Fig. 6A). However, this effect was lost using the high dose (100 μg/ml) of the drug. In line with the DAI scores, DSS-treated mice showed a significant decrease in histologic scores after treatment with the low dose (12.5 μg/ml) of nicotine. However, this effect started to decrease gradually with increasing doses (25 and 100 μg/ml) of nicotine (Fig. 6B). Furthermore, oral administration of nicotine at doses of 12.5 and 25 μg/ml normalized the appearance of the epithelial architecture of the colonic slices and reversed the DSS-induced colonic damage (Fig. 6, E and F).

To determine whether nicotine is bioavailable after oral administration in our treatment protocols, the plasma levels of the drug and its main metabolite were measured using the

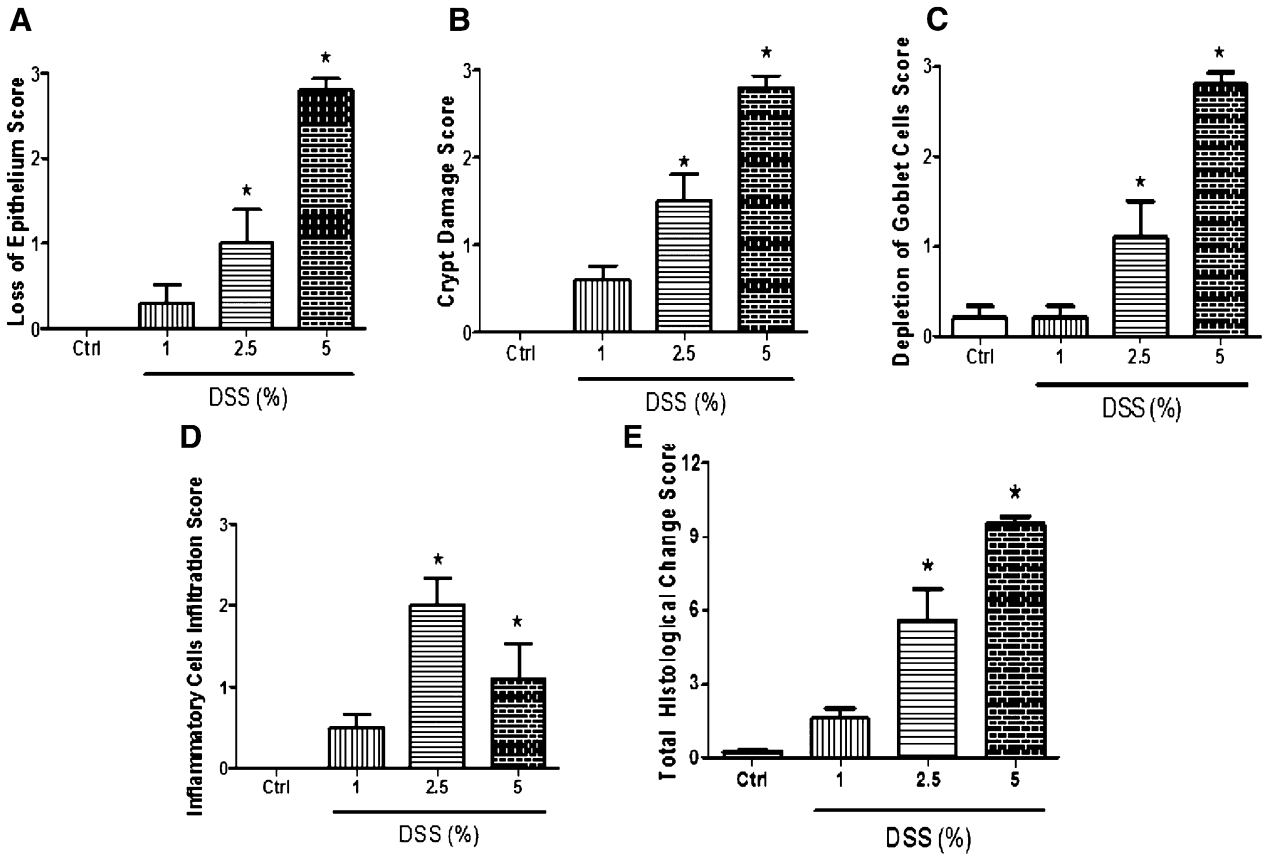

Fig. 3. Histologic scores from colons after 7 days of exposure to water or different doses of DSS. (A) Loss of epithelium. (B) Crypt damage. (C) Depletion of goblet cells. (D) Infiltration of inflammatory cells. (E) Total histologic damage score. Histologic scores were calculated blindly, using H&E-stained colonic tissue sections, after 7 days in the water-treated control group (Ctrl) and DSS-treated animals. Results are expressed as mean ± S.E. (n = 3). *$P < 0.05$.

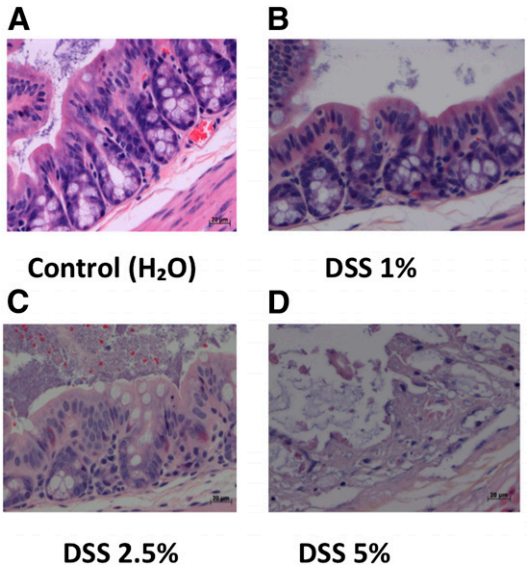

Fig. 4. Histologic analysis of representative colons from mice after 7 days of exposure to water or different doses of DSS. (A) Appearance of colon from water-treated control. (B) Appearance of colon treated with 1% DSS. (C) Appearance of colon treated with 2.5% DSS. (D) Appearance of colon treated with 5% DSS. H&E stain; original magnification, 40×. Scale bar, 20 μm.

lowest active dose and the highest dose of nicotine in DSS-treated mice. Oral nicotine administration resulted in a dose-related increase in the plasma levels of nicotine and cotinine (Table 1) in DSS-treated mice.

Effect of Subcutaneous Nicotine Treatments on DSS-Induced Colitis. Subcutaneous nicotine treatment (0.1, 0.5, and 2 mg/kg s.c. twice daily for 10 days) was evaluated in the DSS model. Overall, nicotine given s.c. failed to significantly alter the DAI in the DSS-treated animals. Only the low dose of 0.1 mg/kg s.c. resulted in a significant decrease in the DAI on days 7 and 8 (Fig. 7A). A decrease in the AUC was observed with the same dose, but it failed to reach statistical significance (Fig. 7B) [$F(3,36) = 2.604$, $P = 0.0668$)]. Similarly, only mice injected with 0.5 mg/kg s.c. showed a significant attenuation in the colon length shortening compared with the vehicle DSS-treated group [$F(4,42) = 20.76$, $P < 0.05$] (Fig. 7C). However, none of the nicotine doses tested after chronic s.c. injection resulted in a significant decrease in total histologic damage score compared with DSS-treated mice (Fig. 7D). Similarly, nicotine did not reverse colonic TNF-α levels in DSS-treated mice (Fig. 7E). Finally, chronic administration of 0.5 and 0.1 mg/kg nicotine increased the plasma levels of nicotine and cotinine in mice. Drug plasma levels after administration of 0.1 mg/kg nicotine were below the level of detection (Table 1).

Effect of Chronic Nicotine Infusion on DSS-Induced Colitis. Chronic exposure of mice to nicotine via minipump infusion at doses of 2.5 or 25 mg/kg per day was assessed. Overall, nicotine given via infusion failed to significantly alter the colitis severity in DSS-treated animals. Only the low dose of 2.5 mg/kg per day resulted in a significant decrease in the DAI on day 5 compared with the control group (Fig. 8A). However, no significant decrease in the AUC in nicotine-treated mice was seen at either infused dose (Fig. 8B). Similarly, nicotine

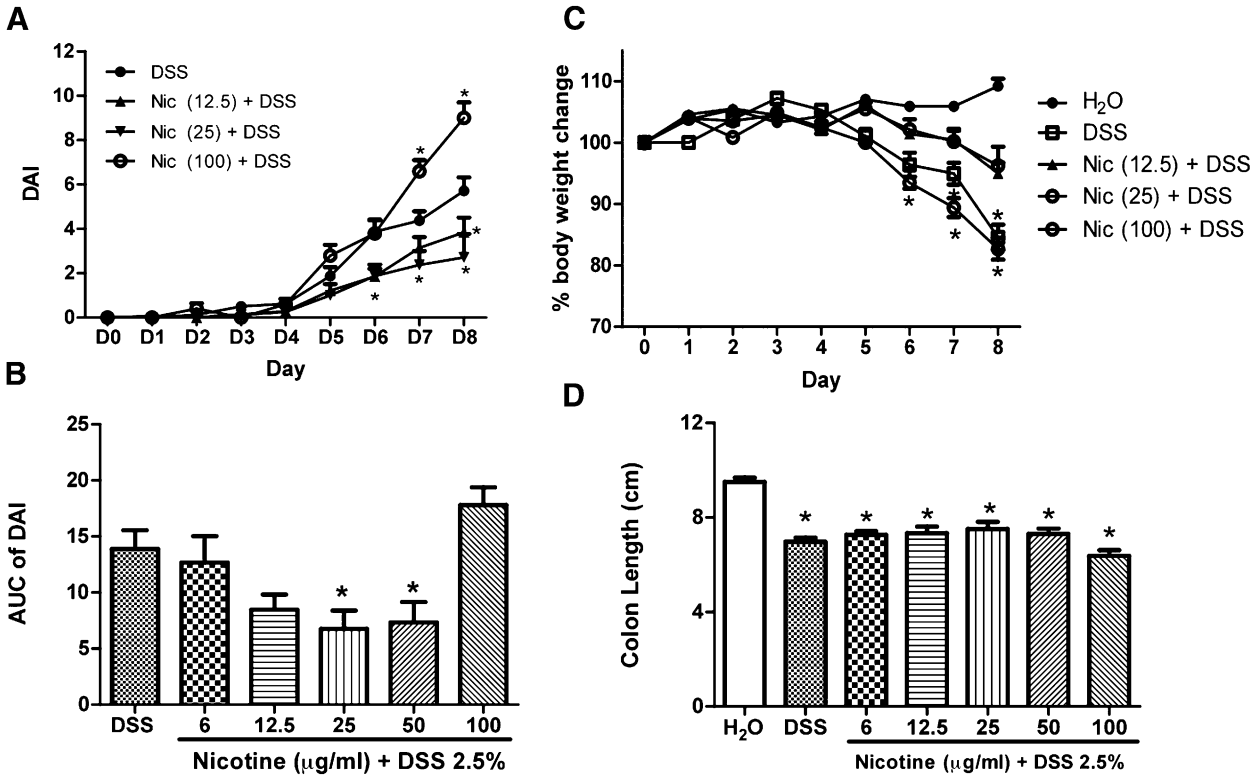

Fig. 5. Oral nicotine treatment suppressed the severity of DSS-induced colitis in mice. Effects of various doses (6–100 μg/ml) of chronic oral nicotine (Nic) treatment on the time course of DAI (A), AUC of DAI (B), percentage of body weight change (C), and mean colon length (cm) (D). Results are expressed as mean ± S.E. (n = 6–8). *$P < 0.05$, compared with control (vehicle-treated mice).

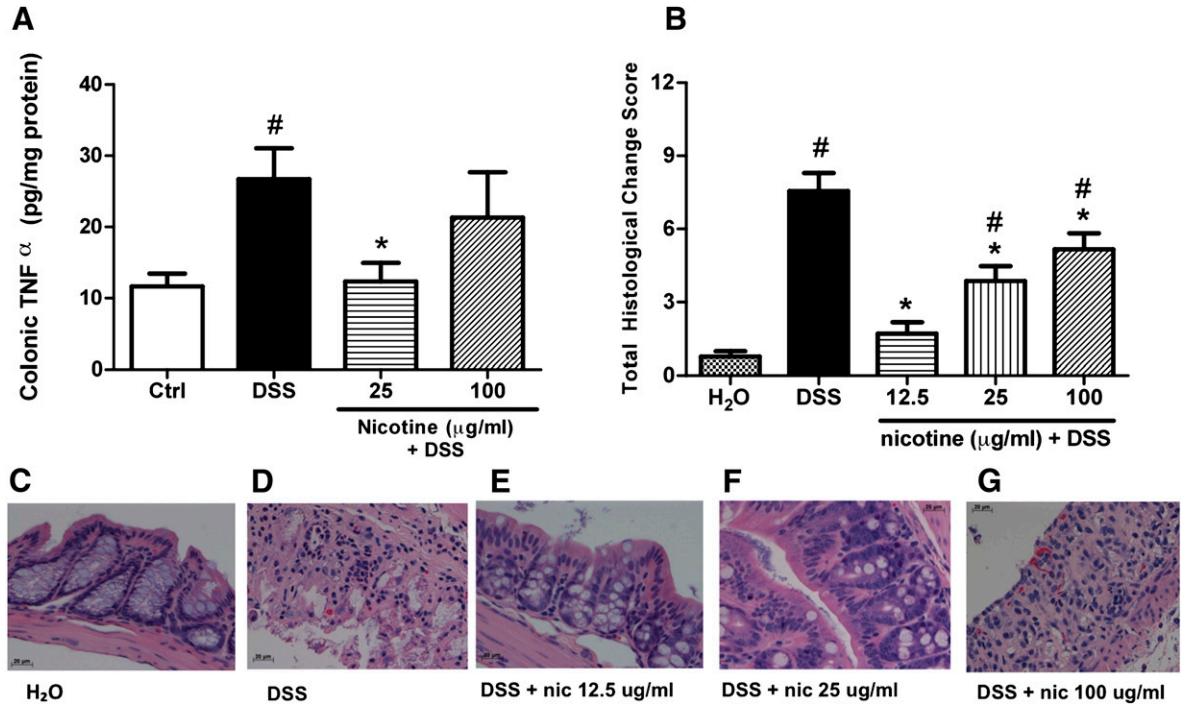

Fig. 6. Oral nicotine treatment (25 and 100 μg/ml) affects colonic TNF-α levels, histologic damage score, and appearance in DSS-induced colitis. (A) TNF-α levels (pg/mg protein) in homogenized colonic tissue samples. Results are expressed as mean ± S.E. (n = 6–8). (B) Histologic colonic damage score in DSS-induced colitis. Results are mean ± S.E. (n = 3–5 per group). Representative colon sections at 7 days after induction of colitis in a control mouse (C), DSS-treated mouse (D), 12.5 μg/ml nicotine–treated mouse (E), 25 μg/ml nicotine–treated mouse (F), and 100 μg/ml nicotine–treated mouse (G). H&E stain; original magnification 40×. Scale bar, 20 μm. *$P < 0.05$, versus DSS group, #$P < 0.05$, versus water control group. Ctrl, water-treated group; Nic, nicotine.

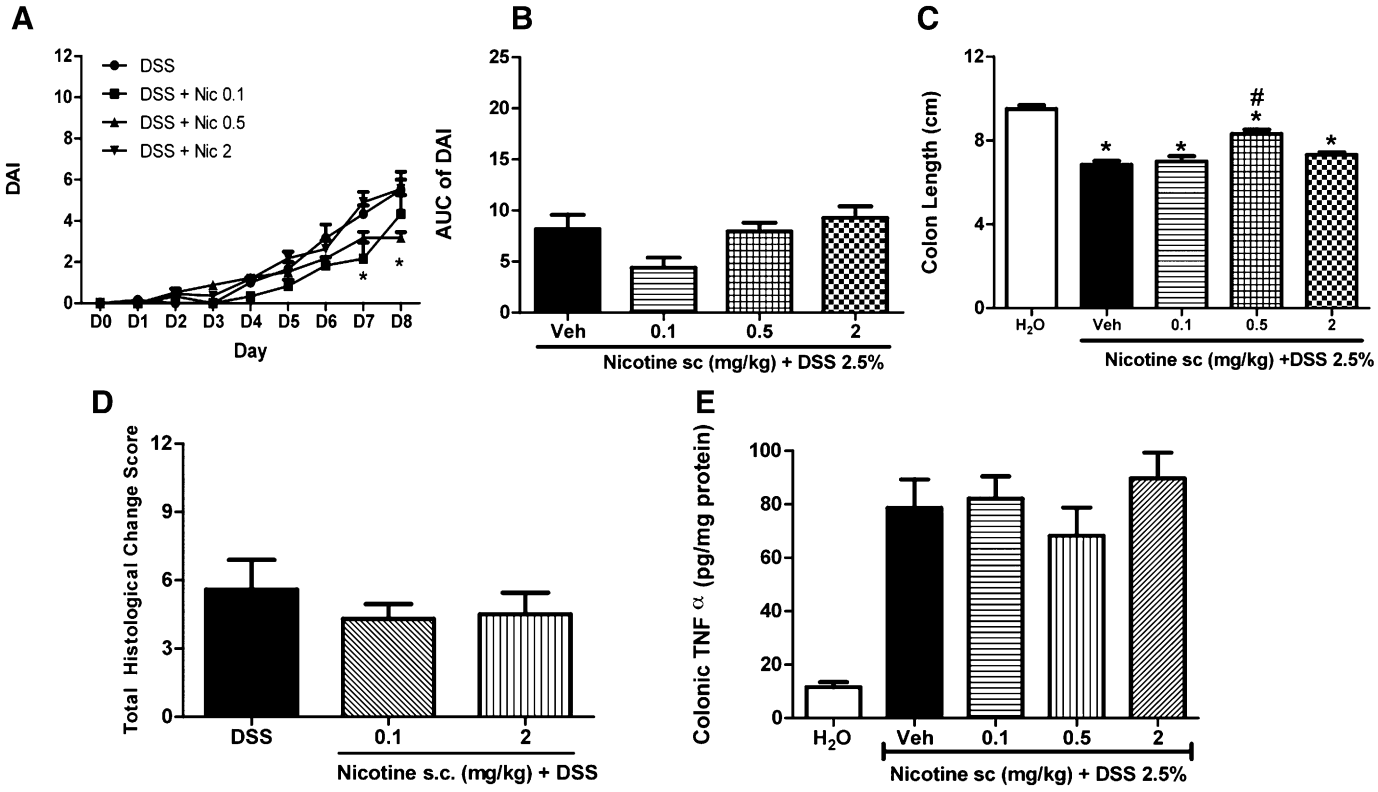

Fig. 7. Effect of chronic s.c. nicotine administration on the severity of DSS-induced colitis in mice. Effects of chronic s.c. nicotine treatment (0.1, 0.5, and 2 mg/kg) on the time course of DAI (A), AUC of DAI (B), mean colon length (cm) (C), and histologic colonic damage score (D). (E) Colonic TNF-α level (pg/mg protein) for mice in (A). Results are expressed as mean ± S.E. (n = 6–8). *$P < 0.05$, versus control (vehicle-treated mice). Nic, nicotine; Veh, vehicle.

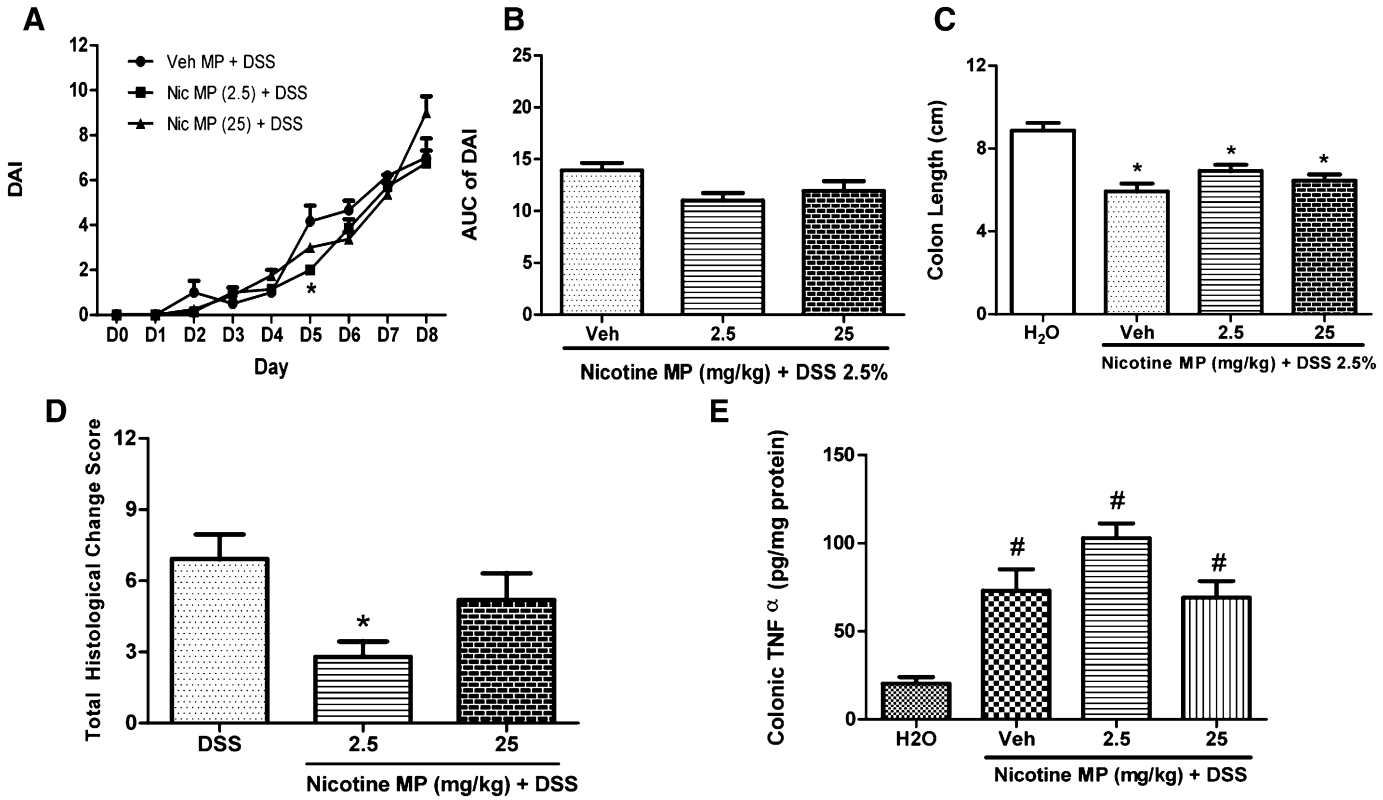

Fig. 8. Influence of nicotine infusion via minipump on DAI, histologic damage, and colon length in DSS-treated mice. Effects of chronic minipump infusion of nicotine (2.5 or 25 mg/kg per day) on the time course of DAI (A), AUC of DAI (B), mean colon length (cm) (C), and histologic colonic damage score (D). (E) Colonic TNF-α level (pg/mg protein) for mice in (A). Results are expressed as mean ± S.E.M. (n = 6–8). *$P < 0.05$, compared with control (vehicle-treated mice). #$P < 0.05$, compared with water group. MP, minipump; Nic, nicotine; Veh, vehicle.

treatment failed to significantly reverse the decrease in colon length in DSS-treated mice (Fig. 8C). However, the low dose of nicotine infusion (2.5 mg/kg per day) resulted in a significant decrease in total histologic damage score compared with DSS-treated mice [$F(2,31) = 4.531$, $P = 0.0188$] (Fig. 8D). This effect was not observed at the high dose (25 mg/kg per day) of nicotine. In contrast, neither dose of nicotine was able to attenuate the increase in colonic TNF-α levels observed in DSS-treated mice [$F(3,46)=16.66$, $P < 0.05$] (Fig. 8E). Finally, chronic infusion of nicotine induced a dose-related increase in the plasma levels of nicotine and cotinine in mice (Table 1).

Effect of Cotinine, the Main Metabolite of Nicotine, in the DSS-Induced Colitis Model. To determine whether cotinine, the principal metabolite of nicotine, possessed anti-inflammatory effects in the colitis model, DSS-treated animals were subjected to chronic cotinine given orally at 25 or 250 μg/ml. As shown in Fig. 9, cotinine administration did not have a significant effect on colitis severity, as measured by DAI scores (Fig. 9, A and B), and it did not result in a shortening of colon length (Fig. 9C). Cotinine was bioavailable because the plasma levels of the drug and its metabolite (3-OHCotinine) (Table 1) were shown to be increased in a dose-related manner after oral administration of the drug at 25 or 250 μg/ml.

Discussion

The objective of this study was to determine the pharmacology of protective effects of nicotine in the DSS-induced colitis mouse model. In addition, we assessed whether cotinine, the main metabolite of nicotine, was effective in this model. The results suggest that low doses of oral nicotine, but not cotinine, are most effective in the colitis model. Our results also show that the dose and route of administration of nicotine play a pivotal role in its effects in the DSS model.

As reported previously (Ahmad et al., 2000; Araki et al., 2000; Egger et al., 2000), exposing male C57BL/6J mice to DSS in the drinking water results in acute inflammation of the colon. Furthermore, in the present study we demonstrated that, as the DSS concentration increases, the severity of disease increases. To further examine the extent of colonic inflammation in animals exposed to DSS, two markers of inflammation, MPO and TNF-α, were examined. MPO is an enzyme in neutrophils, and its activity is used as a marker of inflammation and as an index of neutrophil infiltration (Bradley et al., 1982); TNF-α is an early proinflammatory cytokine mediator that plays a critical pathologic role in colonic inflammation. The activity and/or level of both inflammatory markers had increased significantly after 7 days of DSS treatment in a dose-related fashion.

Our results demonstrated that the dose and route of administration play a pivotal role in nicotine's protective effects in the DSS model of colitis. Although the continuous infusion of a low dose of nicotine (2.5 mg/kg per day) through minipumps resulted in a modest and short improvement in the clinical signs and histologic damage, the effect was not observed with the higher dose of nicotine (25 mg/kg per day). Similarly, chronic s.c. injection of low doses of nicotine (0.1 and 0.5 mg/kg) resulted in a similar improvement in clinical

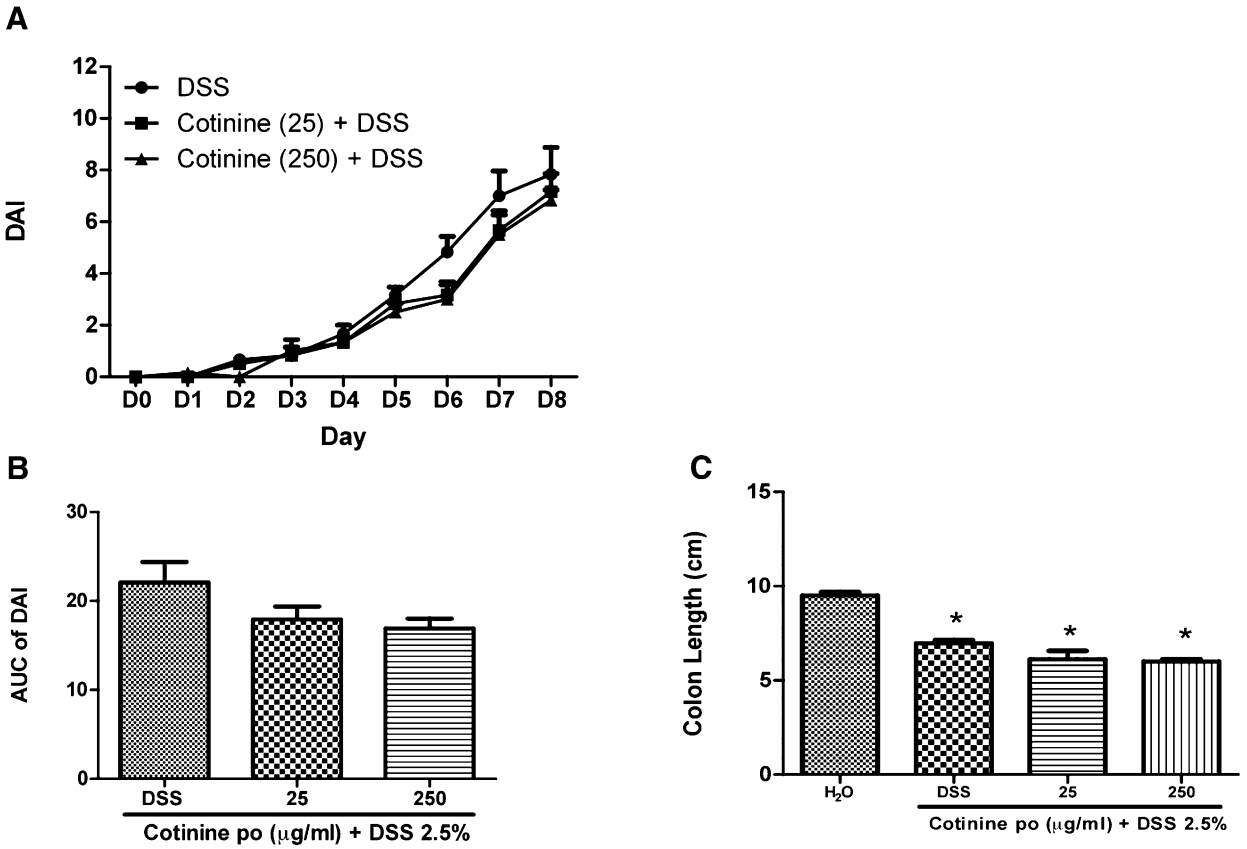

Fig. 9. Lack of suppression of colitis severity by oral cotinine. Effects of oral cotinine (25 or 250 μg/ml) on the time course (A) and AUC (B) of DAI, as well as mean colon length (cm) (C), in mice. Results are expressed as mean ± S.E.M. (n = 6–8). *$P < 0.05$, compared with control (water-treated mice).

signs and in a reversal of the shortening in colonic length. This effect was not observed with the high dose (2 mg/kg) of nicotine. However, neither route of administration effected a reversal in the DSS-induced increase in colonic TNF-α levels. In contrast, oral nicotine treatment attenuated dose-dependently DSS-induced colitis. The low doses of nicotine (12.5–50 μg/ml) exerted a protective effect; they resulted in a reduction in DAI scores, histologic architectural damage, and colonic TNF-α levels. However, the protective effect of nicotine in the DSS colitis model dissipated at the high dose (100 μg/ml). Interestingly, nicotine plasma levels increased only 1.5-fold between the doses of 25 and 100 μg/ml (Table 1) suggesting a narrow concentration-effect profile for oral nicotine in the DSS model. Curiously, oral nicotine treatment at any of the doses tested failed to significantly reverse the shortening in the colon length in the DSS-treated group.

The biphasic effect of chronic nicotine treatment on clinical parameters in DSS-induced colitis in mice was not unexpected. The low doses of oral nicotine (12.5–25 μg/ml) were shown to be protective, whereas the higher doses were not. The low doses administered in drinking water resulted in a reduction in DAI scores, histology architectural damage, and colonic TNF-α levels, whereas the high dose (100 μg/ml) resulted in an exacerbation of DSS colitis, including the DAI score and the AUC. Exacerbation of the DAI score at high doses of nicotine was not observed in s.c. or minipump nicotine-treated animals, suggesting a differential effect of nicotine dose and delivery on colonic inflammation. The results suggesting the protective effects of low-dose nicotine added to the drinking water were similar to those reported in various experimental colitis models in rats and mice (Qiu et al., 1997; Eliakim et al., 1998; 2001; Sykes et al., 2000; Ghia et al., 2006). Qiu et al. (1997) demonstrated that a 5-μg/ml dose of nicotine in the drinking water in 2,4-dinitrobenzene sulfonic acid (DNBS)-treated rats resulted in a significant decrease in intestinal inflammation; however, the inflammation was shown to increase at a dose of 50 μg/ml. Similarly, Eliakim et al. (1998) and Sykes et al. (2000) reported that nicotine had a dual effect on colitis in mice, with low doses of nicotine being more effective at reducing the inflammation than higher doses. This dual anti-inflammatory effect of nicotine in the form of an "inverted-U" was described previously (Picciotto, 2003).

Overall, our results suggest that the route of administration of nicotine is an important factor to consider in the treatment of colitis. The lack of nicotine's clear anti-inflammatory effect after s.c. and minipump infusion in the DSS model could explain some of the inconsistent results that were reported previously in rodent (Snoek et al., 2010) and human studies. Notably, the marginal effects observed after infusion of a constant dose of nicotine through minipumps is consistent with the lack of effects of nicotine using transdermal patches in some UC studies (Pullan et al., 1994; Cosnes, 2004; McGilligan et al., 2007), suggesting that a route of administration that mimics the nicotine-intake profile seen in smokers could result in an increase in the efficacy of treatment. Indeed, these and other studies (Eliakim et al., 1998, 2001, 2002; Sykes et al., 2000; Ghia et al., 2006) suggest

that administering nicotine orally is a very efficacious route for reversing colitis in rodent models. This outcome is consistent with that obtained in UC studies using rectal administration of nicotine. A possible explanation for these results is that nicotine targets and affects the colon locally when administered orally, with marginal therapeutic index. Other possible mechanisms for nicotine's effects have been proposed. For example, the beneficial effects of oral nicotine treatment could be due to it exerting anti-inflammatory effects, in part through changes in colonic TNF-α levels or through inhibition of release of TNF-α and other proinflammatory cytokines from cytokine-producing cells. In the mouse colitis model used in this study, colonic TNF-α levels were reduced significantly with low-dose oral nicotine treatment, which is most likely mediated via suppression of proinflammatory cytokine release from macrophages and other cytokine-releasing immune cells as part of the initial innate-immune response to DSS. Much in vivo and in vitro evidence suggests that α7 nicotinic acetylcholine receptors on the surface of macrophages may mediate the anti-inflammatory effects of nicotine (Rosas-Ballina and Tracey, 2009; Kawahara et al., 2011).

Although the colon may be an important site of action for nicotine following oral administration, the drug likely acts on many peripheral and central inflammatory pathways. Indeed, nicotine is absorbed in the small intestine with low bioavailability (up to 45%) because of a hepatic first-pass effect (Benowitz and Jacob, 1991; Compton et al., 1997; Zins et al., 1997). In the present study, oral administration of nicotine in the drinking water yielded measurable levels of nicotine in the plasma. For example, a plasma nicotine concentration of 18 ng/ml was found after an oral administration of an active dose of 25 μg/ml of the drug.

It was proposed that some nicotine metabolites contribute to the beneficial effect of nicotine in UC patients, because the majority of nicotine is converted to cotinine (~80%) through the cotinine pathway and is present in smokers' blood at much higher concentrations (~250–300 ng/ml) than nicotine (Benowitz et al., 1983; Gori and Lynch, 1985; Benowitz and Jacob, 1994). In the present study, this hypothesis was tested by administering cotinine directly in the drinking water. The results show that oral cotinine administration has no protective effect in the mouse colitis model based on clinical parameters or measurement of colon length. After 10 days of administration of oral cotinine at 25 and 250 μg/ml, plasma concentrations were 90 and 1336 ng/ml, respectively. Because these plasma levels are well above the concentration range of cotinine found after the nicotine-administration protocols used in our various studies, it is possible that lower doses of oral cotinine might have been protective in the DSS model. Overall, the lack of effect of cotinine suggests that, at the doses tested, nicotine's primary metabolite does not mediate the anti-inflammatory effects of nicotine in the mouse colitis model. However, other metabolites, such as nornicotine, which is a component in tobacco, as well as a minor systemic metabolite of nicotine and a nicotinic acetylcholine receptor agonist (Papke et al., 2007), may play a role in the effects of nicotine.

In summary, we demonstrated that chronic oral administration of low doses of nicotine in the drinking water was protective in DSS-induced colitis in mice based on clinical parameters, inflammation markers, and histologic features. However, modest effects were seen after intermittent s.c injection or continuous infusion of nicotine. The collective results highlight that dose and route of administration play a critical role in the protective effect of nicotine in intestinal inflammation. Developing novel pharmaceutical-formulation regimens, such as topical nicotine administration to the colon (enema), could lead to a decrease in the adverse effects seen with systemic nicotine use and ameliorate the clinical efficacy of nicotine in UC conditions.

Authorship Contributions

Participated in research design: AlSharari, Akbarali, White, Licthman, Cabral, and Damaj.

Conducted experiments: AlSharari, Abdullah, Shahab, Auttachoat, and Ferreira.

Performed data analysis: AlSharari, Abdullah, and Damaj.

Wrote or contributed to the writing of the manuscript: AlSharari, Akbarali, White, Licthman, Cabral, and Damaj.

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