Increased severity of experimental colitis in alpha5 nicotinic acetylcholine receptor subunit-deficient mice

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Substantial evidence suggests a negative association between cigarette smoking and the incidence and severity of ulcerative colitis, a common human inflammatory bowel disease. Nicotine has been implicated in this association. The detection of nicotinic acetylcholine receptors in colonic epithelium, the primary tissue affected in ulcerative colitis, suggests a role for these receptors in the beneficial effect of nicotine on colonic inflammation. Using an animal model, we demonstrate for the first time that α5 nicotinic acetylcholine receptor knockout mice have significantly more severe experimental colitis than wild-type controls and that nicotine significantly ameliorates its course when compared with wild-type controls. These findings suggest that α5-containing nicotinic acetylcholine receptors participate in the modulation of colitis in mice, but other nicotinic acetylcholine receptor subunits also mediate the antiinflammatory effects of nicotine. NeuroReport 16:1123–1127 © 2005 Lippincott Williams & Wilkins.

Key words: α5 Subunit; Experimental colitis; Inflammatory bowel disease; Knockout mice; Nicotine; Nicotinic acetylcholine receptors

INTRODUCTION

Ulcerative colitis (UC) is a common inflammatory bowel disease (IBD), whose multifactorial etiology is largely unknown. Substantial evidence supports a negative association between cigarette smoking and UC, which is largely a disease of nonsmokers and exsmokers. Cigarette smoking is associated with a reduced incidence of disease and an attenuated disease course. Nicotine, the principal pharmacologically active component of tobacco smoke, is believed to be responsible for this association [1,2].

Many nicotine-induced effects in humans and animals are mediated through nicotinic acetylcholine receptors (nAChRs). These receptors belong to the large superfamily of ligand-gated ion channels and are expressed throughout both the central nervous system and the peripheral nervous system and in nonneuronal cells [3]. The nAChRs mediate the effects of the endogenous neurotransmitter acetylcholine, and are the principal biological targets of the tobacco alkaloid, nicotine. To date, 12 distinct genes encoding neuronal nAChR subunits have been identified (α2–10, β2–4), generating an abundance of structurally and functionally distinct heteropentameric and homopentameric receptors [4].

nAChR subunits have been detected in the gastrointestinal tract (GIT) of both experimental animals [5,6] and humans [7–9]. Mouse models lacking specific nAChR subunits have demonstrated altered GIT function including hyperperistalsis, stomach and bowel distention and ileal hyperperistaltic or hyperperistaltic responses to nicotine and nicotine agonists, depending on subunit deficiency [10].

On the basis of these findings, we hypothesized a possible association between nAChR subunits and the pathogenesis and course of intestinal inflammation. The administration of dextran sulphate sodium (DSS) induces murine colitis that resembles human UC, and is a reliable model for the study of its pathogenesis [11]. Using DSS, we elicited colitis in α5 and β4 null mutants (α5/−/− and β4/−/−, respectively), and in wild-type (WT) control mice, and the parameters of inflammation were measured. Additionally, we examined the effect of nicotine treatment on the course of colitis in WT and α5 null mice.

MATERIALS AND METHODS

Animals: In all, 120 adult mice weighing 26.26±4.08 g were studied. Mutant mice were homozygous for a null mutation in the α5 or β4 nAChR subunits [12,13]. WT littersmates served as controls. All procedures were approved by the institutional animal and care committee, in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Experimental mice were genotyped before and following the experiment, as described previously [14]. The experimenter was ‘blind’ to the mouse genotype.

Multiplex real-time polymerase chain reaction: Total RNA was isolated from colonic tissue using Tri-Reagent (Sigma, Rehovot, Israel), according to the manufacturer’s instructions. cDNA was synthesized from 1 µg total RNA using
SuperScript II reverse transcriptase (Invitrogen, Carlsbad, California, USA). Multiplex real-time polymerase chain reaction (RT-PCR) of $\alpha_5$ or $\beta_4$ nAChR subunits and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) genes was performed using primers and PCR conditions, as described previously [14].

**Quantitative real-time polymerase chain reaction assay:** To determine the colonic expression level of the $\alpha_5$ and $\beta_4$ nAChR subunit genes, LightCycler FastStart DNA Master SYBR Green 1 (Roche Applied Science, Mannheim, Germany), primer pairs and PCR reaction conditions were used, as described previously [14]. Expression levels of each subunit gene were normalized using Gapdh expression, yielding the relative expression value.

**Induction of experimental colitis:** Colitis was induced by adding 2.5% dextran sulphate sodium (DSS) (molecular weight 40 000; ICN Biomedicals Inc., Aurora, Ohio, USA) to tap water of WT, $\alpha_5$−/− and $\beta_4$−/− mice for 7 days. Control groups for each strain were placed under tap water of WT, $\alpha_5$−/− and $\beta_4$−/− mice for 7 days. Control groups for each strain were allowed to drink ad libitum.

**Nicotine treatment:** Nicotine (12.5 mg/ml) (Sigma) was added to the drinking water of WT and $\alpha_5$-deficient mice for 7 days prior to and for the 7 days of DSS-induction of colitis. In all experiments, animals were allowed to drink ad libitum.

**Observation of colitis:** The daily clinical assessment of mice included measurement of body weight, inspection of stools for diarrhoea, gross blood and occult blood using a guaiac-impregnated diagnostic test (Hemoccult, Smith Kline Diagnostics Inc., San Jose, California, USA). The disease activity index was determined as described previously [15]. The mice were killed on day 7. The entire colon was dissected, measured and weighed. Mucosal samples were processed for determination of mucosal myeloperoxidase (MPO) activity and prostaglandin E2 (PGE2) generation, and tissue samples were obtained for histological assessment.

**Determination of myeloperoxidase activity:** Colonic mucosal scrapings were homogenized in a Polytron (Kinematica GmbH, Krienz-Luzern, Switzerland), as described previously [15]. An aliquot of the supernatant was taken for determination of enzyme activity, as described previously [16].

**Determination of prostaglandin E2 generation:** A 50 mg sample of mucosa was placed in preweighed tubes containing 1.0 ml phosphate buffer (50 mmol/l, pH 7.4). The mucosa was minced and centrifuged in an Eppendorf centrifuge (Hamburg, Germany) for 10 s. The pellet was resuspended in 1.0 ml of the above buffer and incubated for 1 min in a vortex mixer. Ten micrograms of indomethacin was added and tubes were centrifuged for 60 s. The supernatants were kept at −20°C until radioimmunoassays were performed. PGE2 generation was determined using a radioimmunoassay kit, as described previously [17]. The ability of the mucosa to generate PGE2 was expressed as ng/g of wet tissue weight.

**Morphological analysis:** Tissue sections from all experimental animals were obtained from the same areas of the colon. They were fixed in buffered formaldehyde, and 5 μm sections were prepared and stained with haematoxylin and eosin (H&E). Sections were examined and scored by a pathologist who was ‘blind’ to the experimental group. The histopathological changes including depth and extent of the ulcer, presence and extent of inflammation and the location of fibrosis were scored on a scale of 0–4, as described previously [15].

**Statistical analysis:** Statistical significance was determined by two-way analysis of variance (ANOVA), with the different genotypes and the different experimental treatment as the dependent variables. To determine the source of significance, analysis was followed by Student’s t-test or Tukey comparison. For ordinal data, the Kruskal–Wallis test was followed by the Mann–Whitney U-test (SPSS v.12 Inc., Chicago, Illinois, USA). The level of statistically significant differences was defined as p < 0.05. Graphs were constructed with Prism software (Prism, GraphPad Software, San Diego, California, USA).

**RESULTS**

The expression of both $\alpha_5$ and $\beta_4$ nAChR subunits in colonic tissues of WT mice was detected by multiplex RT-PCR analysis (Fig. 1). $\alpha_5$ and $\beta_4$ messenger RNAs were not expressed in colons of $\alpha_5$ and $\beta_4$ knockout mice, respectively, and $\beta_4$ subunit expression was reduced in $\alpha_5$−/− colons. Quantitative RT-PCR analysis supported the effect of
Table 1. Parameters of colitis in untreated, dextran sulphate sodium (DSS) and DSS + nicotine-treated experimental groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wild-type</th>
<th>α5−/−</th>
<th>β4−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of body weight (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSS</td>
<td>−11.3±6.3 (17)</td>
<td>−199.7±89.18</td>
<td>−99±6.2 (8)</td>
</tr>
<tr>
<td>DSS + Nic</td>
<td>−90±10.9 (11)</td>
<td>−10.7±10.4 (15)</td>
<td>NT</td>
</tr>
<tr>
<td>Disease activity (score)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSS</td>
<td>3.0±2.5 (17)</td>
<td>6.1±2.4 (18)</td>
<td>3.8±3.1 (8)</td>
</tr>
<tr>
<td>DSS + Nic</td>
<td>2.5±1.9 (11)</td>
<td>2.9±2.2 (15)</td>
<td>NT</td>
</tr>
<tr>
<td>Colon PGE2 (ng/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1074±95.9 (12)</td>
<td>1409±61.3 (12)</td>
<td>123±104.6 (11)</td>
</tr>
<tr>
<td>DSS</td>
<td>208.6±79.5 (12)</td>
<td>298.7±116.8 (12)</td>
<td>187.8±128.9 (8)</td>
</tr>
<tr>
<td>DSS + Nic</td>
<td>86.2±52.1 (11)</td>
<td>65.4±33.6 (15)</td>
<td>NT</td>
</tr>
<tr>
<td>Colon MPO (units/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.4±0.3 (12)</td>
<td>0.5±0.3 (12)</td>
<td>0.3±0.26 (8)</td>
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<tr>
<td>DSS</td>
<td>1.5±0.8 (16)</td>
<td>2.2±1.2 (16)</td>
<td>1.5±1.1 (8)</td>
</tr>
<tr>
<td>DSS + Nic</td>
<td>1.8±0.7 (11)</td>
<td>1.6±0.5 (14)</td>
<td>NT</td>
</tr>
<tr>
<td>Colon length (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>7.5±0.9 (12)</td>
<td>8.0±0.4 (12)</td>
<td>8.0±0.6 (8)</td>
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<tr>
<td>DSS</td>
<td>5.8±0.6 (10)</td>
<td>5.3±1.2 (10)</td>
<td>6.3±1.1 (8)</td>
</tr>
<tr>
<td>DSS + Nic</td>
<td>6.0±0.5 (11)</td>
<td>5.4±0.6 (15)</td>
<td>NT</td>
</tr>
<tr>
<td>Colon weight (mg/10 cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>198.6±21.5 (12)</td>
<td>217±50.9 (12)</td>
<td>230.4±21.9 (8)</td>
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<tr>
<td>DSS</td>
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<td>366.2±117.4 (18)</td>
<td>415.4±96.5 (8)</td>
</tr>
<tr>
<td>DSS + Nic</td>
<td>318.9±35.9 (15)</td>
<td>318.8±37.1 (15)</td>
<td>NT</td>
</tr>
</tbody>
</table>

Numbers represent mean ± SD. Number of mice tested in each group is in brackets. NT, not tested; Nic, nicotine; DSS, dextran sulphate sodium; WT, wild-type.

α5 subunit deficiency on reduced β4 subunit expression. The relative expression levels of the β4 subunit were more than 10-fold lower in α5−/− colonic tissue than in tissue from WT mice (0.43 and 4.67, respectively).

Study parameters, including colon length and weight, colonic MPO activity and PGE2 generation were not significantly different in WT control mice, α5−/− and β4−/− mice that received tap water throughout the experiment (Table 1).

The addition of 2.5% DSS to drinking water for 1 week induced colonic inflammation in WT, α5−/− and β4−/− mice. The percentage of body weight loss and the disease activity score following DSS administration were similar in WT and β4 null mutants (Table 1). The percentage of body weight loss was significantly greater in α5−/− mice than in WT and β4 null mutants (p<0.01, ANOVA followed by Tukey comparison, Table 1). Similarly, the disease activity score was significantly greater in α5 null mice than in WT and β4 null mutants (p<0.01 and p<0.05, respectively, Kruskal–Wallis followed by Mann–Whitney U-test). Colonic shortening and the increased wet weight of colonic segments following 1 week of DSS treatment were significant in all groups tested (p<0.001, two-way ANOVA, Table 1). Colonic shortening was greater in DSS-treated α5−/− mice than in DSS-treated β4−/− mice (p=0.061, Student’s t-test, Table 1).

DSS treatment significantly altered the colonic MPO activity and PGE2 levels in all mice groups (p<0.001, two-way ANOVA, Table 1). MPO activity was much greater in α5−/− mice than in WT controls (nearly statistically significant, p=0.057). PGE2 levels were significantly higher in α5−/− mice than in WT mice and nearly statistically significant when compared with β4−/− mice (p=0.05 and p=0.061, respectively, two-way ANOVA followed by Student’s t-test).

Histological scoring of colonic sections confirmed the difference in the severity of DSS-induced colitis in WT and α5−/− mice. Following 1 week of DSS administration, the mean histological score in α5−/− mice was approximately 35% higher than the mean histological score in WT littermates (8.87±2.5 and 6.64±2.79, respectively, p<0.05, Kruskal–Wallis followed by Mann–Whitney U-test). The colons of DSS-treated WT mice exhibited mild architectural distortion of glands with minimal submucosal inflammation (Fig. 2a). Injury in α5−/− mice involved the entire thickness of the bowel wall, with fissingure ulcers and moderate inflammatory infiltrate in the mucosa, submucosa and the inner muscular layer (Fig. 2b).

In a follow-up experiment, the effect of nicotine on the course of DSS-induced colitis was evaluated in WT and α5−/− mice. When compared with α5 null mice that did not receive nicotine, the severity of DSS-induced colitis was attenuated in nicotine-treated α5 null mice, as reflected by the significantly reduced disease activity score (p<0.001, Mann–Whitney U-test, Table 1). In contrast, nicotine did not have a significant effect on the disease activity score in WT mice (Table 1). Similarly, the loss of body weight in α5−/− mice treated with DSS and nicotine was significantly less than body weight loss induced by DSS alone (p<0.01, Student’s t-test, Table 1). The addition of nicotine did not significantly reduce DSS-induced weight loss in WT mice.

PGE2 generation was significantly reduced both in WT and in α5−/− mice treated with nicotine and DSS when compared with animals treated with DSS alone (p<0.001, two-way ANOVA). This reduction, however, was more than two-fold greater in α5 null mice than in WT mice (Table 1). Nicotine treatment also decreased the colonic wet weight in both α5−/− and WT mice compared with mice treated with DSS alone (p=0.057, two-way ANOVA, Table 1). Nicotine treatment did not alter MPO activity when compared with...
mice treated with DSS alone, nor did it result in a significant increase in colonic length in $\alpha_5^--/-$ or WT mice (Table 1).

DISCUSSION

We have demonstrated, for the first time, a role for a specific nAChR subunit, the $\alpha_5$ subunit, in the modulation of intestinal inflammation in an animal model.

The $\alpha_5$ nAChR subunit is expressed throughout both the central nervous system and the peripheral nervous system [12]. Its expression has also been detected in nonneuronal cells, including the thymus, epidermal and oral keratinocytes, bronchial epithelium and throughout the GIT [7,18], specifically on human colonic epithelium [19].

$\alpha_5$ is an auxiliary subunit, which forms functional ion channels only when coexpressed with both $\alpha$ and $\beta$ subunits. The addition of $\alpha_5$ to functioning doublet subtypes, such as $\alpha_4\beta_2$ and $\alpha_3\beta_2$ or $\alpha_3\beta_4$, modifies the pharmacological and biophysical properties of the nAChR. Altered nicotine-gated conductance, desensitization rate, Ca$^{2+}$ permeability, and affinity and sensitivity of certain nicotinic agonists and antagonists vary, depending on the nature of the subunits coexpressed with $\alpha_5$ [4,10,12,20].

In the current study, the exacerbated inflammation witnessed in $\alpha_5^--/-$ mice suggests that coassembly of the $\alpha_5$ subunit in GIT nAChRs reduces the susceptibility to, or the severity of, experimental colitis in mice, possibly owing to the influence of this subunit on intestinal structure and function. Acetylcholine-induced nAChR-mediated epithelial stability has been demonstrated in bronchial epithelium and keratinocytes [18]. A similar mechanism, possibly involving $\alpha_5$-containing nAChRs, might also occur in colonic epithelium.

The administration of nicotine attenuated the severity of colitis in both WT and $\alpha_5$ null mice. Substantial evidence from human and animal studies supports the therapeutic effects of smoking and transdermal nicotine in ulcerative colitis, although the mechanisms of action are complex and only partially understood. Changes in gut motility, colonic mucin synthesis, eicosanoid-mediated inflammation and the production of proinflammatory cytokines likely have a role in nicotine-induced attenuation of colonic inflammation [1,2]. nAChR-dependent mechanisms might also contribute to the antiinflammatory effects of nicotine. A 'cholinergic antiinflammatory pathway' has been identified, whereby the synthesis and release of the central inflammatory mediator tumour necrosis factor from macrophage cells is inhibited through the activation of nAChRs assembled from the $\alpha_7$ subunit. This pathway is believed to attenuate systemic inflammatory responses, including those of the GIT [21]. Moreover, the recent detection of nAChRs on colonic epithelium [9,19], the primary tissue affected in UC, strengthens the suggestion that nicotine-induced attenuation of colitis is to some extent nAChR-mediated.

Interestingly, the favourable response to nicotine was more significant in $\alpha_5^--/-$ mice, mice lacking the potentially GIT-protective $\alpha_5$ subunit. The deficiency of the $\alpha_5$ subunit likely elicits modified nAChRs, possibly altering an $\alpha_3$-containing native triplet receptor (such as $\alpha_3\beta_4\delta$ or $\alpha_3\beta_5\delta$) to functioning doublet ($\alpha_3\beta_4$ or $\alpha_3\beta_2$), subtypes that might be more sensitive to the therapeutic antiinflammatory effects of nicotine. Studies in knockout mice have already shown that nAChR subunit deficiency may alter receptor function and sensitivity to ligands [4,10]. In $\alpha_5^-/-$ mice, ileal contractile response [20] and autonomic presynaptic nAChR activation [22] were elicited by nicotinic agonists with a much higher efficacy than in WT controls, suggesting that the absence of $\alpha_5$ increases receptor sensitivity to certain nicotinic agonists.

CONCLUSION

On the basis of the study of experimental colitis in WT, $\alpha_5^-/-$ and $\beta_4^-/-$ mice, we suggest that $\alpha_5$-containing receptors are involved in the modulation of colonic inflammation. Additionally, our findings suggest that the nicotine-induced antiinflammatory activity associated with colitis likely involves an nAChR-mediated mechanism, and is dependent on the yet unidentified composition of specific nAChR subunits.

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