Side-stream smoking reduces intestinal inflammation and increases expression of tight junction proteins

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Abstract

AIM: To investigate the effect of side-stream smoking on gut microflora composition, intestinal inflammation and expression of tight junction proteins.

METHODS: C57BL/6 mice were exposed to side-stream cigarette smoking for one hour daily over eight weeks. Cecal contents were collected for microbial composition analysis. Large intestine was collected for immunoblotting and quantitative reverse transcriptase polymerase chain reaction analyses of the inflammatory pathway and tight junction proteins.

RESULTS: Side-stream smoking induced significant changes in the gut microbiota with increased mouse intestinal bacteria, *Clostridium* but decreased *Firmicutes* (*Lactococi* and *Ruminococcus*), *Enterobacteriaceae* family and *Segmented filamentous baceteria* compared to the control mice. Meanwhile, side-stream smoking inhibited the nuclear factor-κB pathway with reduced phosphorylation of p65 and IκBα, accompanied with unchanged mRNA expression of tumor necrosis factor-α or interleukin-6. The contents of tight junction proteins, claudin3 and ZO2 were up-regulated in the large intestine of mice exposed side-stream smoking. In addition, side-stream smoking increased c-Jun N-terminal kinase and p38 MAPK kinase signaling, while inhibiting AMP-activated protein kinase in the large intestine.

CONCLUSION: Side-stream smoking altered gut microflora composition and reduced the inflammatory response, which was associated with increased expression of tight junction proteins.

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Key words: Inflammation; Microbiota; Tight junction protein; Side-stream smoking; Intestine

INTRODUCTION

Cigarette smoking is a remarkable etiological factor in the pathogenesis of cardiovascular diseases, hypertension, pulmonary diseases and gastroenterological diseases[1-4]. Meanwhile, passive smoking (second-hand smoking) is also a contributing factor for the development of coronary artery disease[5-7], lung cancer[7] and Crohn’s disease[8], which pose a substantial health risk to non-smoking adults and young children worldwide[9]. It was estimated in 2004 that more than 600 thousand deaths were due to...
second-hand smoke, which accounted for about 1% of worldwide mortality. On the other hand, it was reported that smoking had a protective effect in reducing ulcerative colitis mostly based on the epidemiologic studies.

Chronic inflammatory bowel diseases, mainly Crohn’s disease and ulcerative colitis, are characterized by chronic inflammation of the intestines. Recent studies clearly show that gut epithelial integrity and barrier function are the central predisposing factors in inflammatory bowel diseases, autoimmune and related allergic diseases. The intestinal epithelium is composed of tightly assembled intestinal epithelial cells which form a protective barrier against pathogenic and commensal bacteria, preventing their penetration from the lumen to initiate inflammatory responses in the mucosal system. Impairment of the tight junction barrier is associated with chronic diseases such as inflammatory bowel diseases, obesity and type 1 diabetes. Epithelial cells form an integrated web through interaction of tight junction proteins including intracellular proteins, zona occludens, and membrane proteins, occludin, claudin and junctional adhesion molecules. The tight junction functions are affected by extracellular stimuli such as the microbial components, pro-inflammatory cytokines and stress. Inflammation disrupts tight junctions. Inflammatory cytokines such as interleukin (IL)-13, and IL-6, increase tight junction permeability through increasing claudin 2 expression. The activation of the inflammatory pathway nuclear factor (NF)-κB by TNF-α, down-regulates ZO-1 gene expression and induces its relocation in Caco-2 cells. Therefore, local inflammation impairs the barrier function of gut epithelium.

The “microflora hypothesis” suggests that gut microflora composition plays an important role in the immunological response of the gut. Lactic acid bacteria are known to have an anti-inflammatory effect, and alteration of microflora composition is linked to the incidence of inflammatory bowel diseases. Up to now, there is no published studies assessed gut microflora changes due to smoking. We hypothesized that side stream smoking may possess a potent anti-inflammatory effect on the gut mucosal immune system which promotes the expression of tight junction proteins in the intestine, exerting beneficial effects on the prevention of ulcerative colitis.

**MATERIALS AND METHODS**

**Animal care and experiment design**

C57BL/6 female mice at 6 mo of age were housed in a temperature-controlled room with a 12 h light and 12 h darkness cycle and were given food and water ad libitum. Mice were placed in an exposure box and exposed to side-stream smoke for 1 h daily for 40 d. Commercial cigarettes (golden monkey, tar: 13 mg; nicotine: 1.1 mg; CO: 15 mg) were used at a dose equivalent to one commercial cigarette’s smoke per day. The animal care procedures described in this study was approved by the University of Wyoming Institutional Animal Use and Care Committee.

**Tissue collection**

On the day of necropsy, mice were anesthetized intra-peritoneally with tribromoethanol (250 mg/kg body wt). Blood samples were collected from the orbital sinus while mice were under general anesthesia. Mice were then sacrificed by cervical dislocation. Large intestines were dissected, flushed with phosphate-buffer saline and then frozen in liquid nitrogen for immunoblotting and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses. Cecal contents from each mouse were collected and frozen for microflora analyses.

**Reagents and antibodies**

Antibodies against ZO1, ZO2, Claudin3 and Occludin were purchased from Invitrogen (Camarillo, CA). Antibodies against phospho-ε-Jun N-terminal kinase (SAPK/JNK) (Thr183/Tyr185), SAPK/JNK, phospho-NF-κB p65 (ser536), NF-κB p65, phospho-IκB kinase (IKK) α/β (Ser176/180), IKKβ, phospho-IκBα, IκBα, phospho-p38 MAP kinase and p38 MAP kinase, phospho-AMP-activated protein kinase (AMPK) α and AMPKα were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against xanthine oxidase (XO), heat shock protein (HSP) 60 and superoxide dismutase (SOD) 1 were purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Affinity BioReagents (Golden, CO).

**Quantitative reverse transcription PCR**

Total RNA was extracted from powdered large intestine using Trizol® Reagent (Sigma, St. Louis, MO), treated with DNase I (Qiagen, Valencia, CA) and purified with RNeasy Mini kit (Qiagen). cDNA was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). qRT-PCR was conducted on a Bio-Rad CFX96 machine (Bio-Rad Laboratories, Hercules, CA) and purified with RNeasy Mini kit (Qiagen). cDNA was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). qRT-PCR was conducted on a Bio-Rad CFX96 machine and SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) was used for all qRT-PCR reactions. Mouse GAPDH was used as the housekeeping gene. Primer sequences are listed in Table 1. The final primer concentration was 200 nM for each gene. The amplification efficiency was 0.90-0.99. The qRT-PCR conditions were 95 °C, 3 min, and 35 cycles of 95 °C for 10 s, 58 °C for 20 s and elongation step at 72 °C for 20 s. At the end of each run, dissociation melting curve was obtained to confirm the purity of PCR products.

**Microflora analyses**

The frozen caecal contents (0.1 g) were homogenized and bacterial genomic DNA was extracted using a QIAamp DNA stool mini kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). The abundance of specific intestinal bacterial groups was measured by qPCR using Bio-Rad CFX96 machine (Bio-Rad Laboratories, Hercules, CA) as described above. Group specific
or kingdom specific 16S rRNA gene primers were listed in Table 2. Eubacteria 16S rRNA was used as the housekeeping gene.

**Immunoblotting analyses**

Immunoblotting analyses were conducted as previously described\(^{[39,40]}\). Briefly, protein extracts from the mouse large intestine were separated by 5%-15% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gradient gels and transferred to nitrocellulose membranes for immunoblotting analyses. Band density was normalized according to the GAPDH content\(^{[39,40]}\).

**Statistical analysis**

Statistical analyses were conducted as previously described\(^{[41-43]}\). Data were analyzed as a complete randomized design using General Linear Model of Statistical Analysis System (2000). Mean ± SEM are reported. Mean difference was separated by a least significant difference multiple comparison test. Statistical significance is considered as \(p < 0.05\).

**RESULTS**

**Effect of side-stream cigarette smoking on the gut microflora composition**

Quantitative PCR analysis of 16S rRNA showed that exposure of C57BL6 mice to side-stream cigarette smoking increased the amount of Clostridium clostridiforme and mouse intestinal bacteria (MIB) in the cecal microflora, while decreasing the content of Lactoccoci, Ruminococcus albus, Enterobacteriaceae and segmented filamentous bacteria.

**IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α; ZO: Zona occludens; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.**

**Table 1** Primer sets used for quantitative reverse transcriptase polymerase chain reaction of mouse large intestine tissue

| Gene name | Accession no. | Product size | Direction | Sequence (5' → 3') | Source |
|-----------|---------------|--------------|-----------|--------------------|--------|
| IL-6      | NM_031168.1   | 107 bp       | Forward   | GCTGGTGACAACACGCCGCT | This study |
| TNF-α     | NM_013693.2   | 67 bp        | Forward   | AGCCCTCCAGCTTGAAGCTTGTT | [58] |
| Claudio 3 | NM_009802.4   | 132 bp       | Reverse   | CAGGGCGACTCTGCTGCGAG | This study |
| Occludin  | NM_008756.2   | 308 bp       | Reverse   | ATGTCGCCGCGATCTCTGTC | [58] |
| ZO-1      | NM_009386.2   | 403 bp       | Reverse   | AATGGCCCGCGACACCTTGCTGA | [58] |
| ZO-2      | AF113005.1    | 106 bp       | Reverse   | GCCAGCAACAGGACACCTTCTCAA | This study |
| GAPDH     | NM_008084.2   | 132 bp       | Reverse   | GATGCGAGGTCGTAGTCTTCTTGA | This study |

**Table 2** Primer sets used for quantitative polymerase chain reaction of 16S rRNA of specific bacterial species or genus

| Target organism | Primer set | Sequence (5’ to 3’) | Product size | Annealing temp (°C) | Reference |
|-----------------|------------|---------------------|--------------|---------------------|-----------|
| Bacteroides     | BactF285   | GGTTCTGAGAGGAGGTCCC | 53           | 61                  | \[59\]    |
|                 | UniR338    | GTCGGTCTTCCGTCAGGAGT | 61           |                     |           |
| Clostridium butyricum | BactF285   | GGTTCTGAGAGGAGGTCCC | 53           | 61                  | \[59\]    |
|                 | UniR338    | GTCGGTCTTCCGTCAGGAGT | 61           |                     |           |
| Clostridium clindiforme | Cclos99F  | AATCTTGATTGACTGAGTGGCGGAC | 148         | 62                  | \[60\]    |
|                 | Cclos247R  | CATCCTCACACTGCCGCTGTTTTC | 62           |                     |           |
| Clostridium perfringens | Cperf165F | CGCATAACGTTAGGAAGATGG | 104         | 61                  | \[59\]    |
|                 | Cperf269R  | CTCGTTAGGCCGTTACC | 61           |                     |           |
| Enterobacteriaceae | Eco1457F  | CATTGAGGTTATGCAAGAACGC | 195         | 63                  | \[60\]    |
|                 | Eco1652R  | CTTCAAGGATGAACTGTCG | 63           |                     |           |
| Enterooccus     | Ec-ssu1F   | GGATAAACACTGTTAGAACG | 115         | 60                  | \[61\]    |
|                 | Ec-ssu1R   | TCTGTTTCTTCCTCACACAA | 60           |                     |           |
| Eubacteria      | UniF340    | ACTCTACTCGGGGAGGCACAGT | 210         | 63                  | \[62\]    |
|                 | UniR514    | ATTACCGCCGCTTGCTGGC | 63           |                     |           |
| Facalibacterium prausnitzii | Fprau223F | GATGCGCTCTGCGGGCATTAG | 199         | 58                  | \[60\]    |
|                 | Fprau428R  | CCGGAGACCTCCTCTCCTCC | 58           |                     |           |
| Lactobacili    | LabF626    | ACCAGTGGAGGAAATCCTCA | 315         | 56                  | \[59\]    |
|                 | LabB677    | CACCGTCTACAGATGGAG | 56           |                     |           |
| Mouse intestinal Bacteria | Uni516F  | CCAGGGAGGCCGCTTAAAT | 161         | 58                  | \[59\]    |
| Segmented filamentous bacteria | SFB736F  | GACGCTGAGGCAGCATGACGACAT | 108         | 58                  | \[58\]    |
| Raminoceccus albus | SFB844R  | GACGGAGCCGAGATTTCACATA | 58           |                     |           |
|                 | Rab856F    | CAGTGCTGAAATTATCGACGG | 246         | 63                  | \[60\]    |
|                 | Rab860R    | GTCACTGCTTCCTTCACACCTTAC | 63           |                     |           |
Intestinal inflammatory responses of gut to side stream smoking

Side-stream smoking decreased phosphorylation of NF-κB p65, a key mediator of the NF-κB inflammatory signaling pathway. Consistently, phosphorylation of IκBα and IKKα/β were also down-regulated in mice exposed to side-stream smoking, indicating that smoking is capable of reducing inflammation in the gut (Figure 2). qRT-PCR analysis indicated that mRNA expression of the two main inflammatory cytokines, TNFα and IL-6, were not changed (data not shown).

Side-stream smoking induced oxidative stress in large intestine

There was an enhanced oxidative stress in side-stream smoking mice compared to that of control mice, as indicated by increased XO ($p = 0.06$) and decreased SOD1 ($p < 0.01$) protein content in the side-stream smoking mice (Figure 3). Meanwhile, the heat shock protein 60 (HSP60) decreased in the side-stream smoking mouse large intestine when compared to that of control mice (Figure 3). Consistently, the phosphorylation of stress signaling mediators, JNK and p38 MAP kinase, were increased in the large intestine of side-stream smoking mice (Figure 4). However, the phosphorylation of another kinase related to stress, AMPK, was reduced in response to side-stream smoking (Figure 5).
increased in the large intestine of side-stream smoking mice (Figure 6B), while there is no difference in their mRNA expression (Figure 6A).

DISCUSSION

Epidemiology studies have shown that smoking, including passive smoke inhalation, reduces the incidence of ulcerative colitis, which may be due to the reduction of epithelial permeability[44]. Intestinal permeability was reduced in healthy smokers compared to the non-smokers[45,46].

Mechanisms by which side-stream smoking improves intestinal tight junctions are not well understood. Previous studies suggest that activation of NF-κB signaling increases intestinal permeability[47]. In this study, we observed that the NF-κB signaling was down-regulated in mice exposed to side-stream smoking. This indicates that side-stream smoking negatively regulates NF-κB signaling which might be a contributing factor to the reduction of intestinal permeability. We also observed that side-stream smoking increased Claudin3 and ZO-2 content without affecting Occludin and ZO-1. In summary, our data revealed that side-stream smoking up-regulated the expression of tight junction proteins and inhibited NF-κB signaling, which may be responsible for the preventive effect of smoking on ulcerative colitis.

Smoking generates reactive oxygen species and nitrogen species in blood, resulting in oxidative stress[48-50]. In this study, we also observed that oxidative stress related enzymes such as xanthine oxidase and superoxide dismutase 1 were altered in the large intestine due to side-stream smoking. Consistent with altered oxidative stress, two pivotal stress signaling mediators, the activation of JNK and p38 signaling were enhanced in the large intestine of mice exposed to side stream smoking. Previously, it was reported that oxidative stress related signaling promotes tight junction protein claudin1 expression in hepatocytes and Sertoli cells[51,52].

A recent published study in gut epithelial cells shows that AMPK is related to the impairment of tight junction and barrier properties of gut induced by inflammation[53]. Our data showed that AMPK activity was dramatically inhibited in the gut tissue of side-stream smoking mice, which may provide an additional mechanism for the association between passive smoking and gut epithelial barrier function.

Furthermore, we found that microflora were altered due to the side-stream smoking. The “microflora hypothesis” suggests that gut microflora composition plays an important role in the immunological response of the gut[29]. Up to now, there have been no published studies assessing changes in gut microflora due to smoking. Our data showed that exposure to side-stream smoking altered the composition of cecal microflora, reducing Firmicutes.
that side stream smoking may possess potent anti-inflammatory effects, which inflammation impairs the barrier function of gut epithelium. We hypothesized function is a central predisposing factor to inflammatory bowel diseases. Local of inflammatory gastrointestinal diseases. Gut epithelial integrity and barrier

Despite its apparent harmful effects, side-stream smoking reduces the risk

**Background**

and *Enterobacteriacea*. Both *Firmicutes* and *Enterobacteriacea* belong to a group of bacteria contributing to fermentation and nutrient intake. *Lactococci* and other lactic acid bacteria are known to have anti-inflammatory effects. The dramatic reduction of *Lactococci* in side-stream smoking mice indicates that *Lactococci* might not be responsible for the reduced inflammation in the gut of side-stream smoking mice. The reason for the reduction of *Lactococci* in cecal microflora due to smoking is unclear, but might be related to oxidative stress. Many *Lactococci* lack catalase and are sensitive to oxidative stress, which may render them less competitive in the oxidative environment induced by smoking. We also observed that MIB was increased while SFB was decreased in smoking mice. Because SFB is known to have important roles in maturation of the gut immune system, its reduction in smoking mice could be associated with the adverse effect of smoking on Crohn’s disease. MIB refers to a group of bacteria called *Corynebacterium-Flavobacterium-Bacteroides* phylum, and their abundance in the gut is known to be altered by environmental factors. The biological effect of MIB alteration due to smoking is unclear.

In conclusion, data from our present study demonstrated that exposure to side-stream smoking inhibited mucosal inflammation and enhanced the expression of tight junction proteins in the large intestine. Further, side-stream smoking increased oxidative stress and altered gut microflora composition.

**COMMENTS**

**Background**

Despite its apparent harmful effects, side-stream smoking reduces the risk of inflammatory gastrointestinal diseases. Gut epithelial integrity and barrier function is a central predisposing factor to inflammatory bowel diseases. Local inflammation impairs the barrier function of gut epithelium. We hypothesized that side-stream smoking may possess potent anti-inflammatory effects, which promote the expression of tight junction proteins in the intestine, exerting beneficial effects on the prevention of ulcerative colitis.

**Research frontiers**

Epidemiologic studies indicate that smoking had a protective effect on ulcerative colitis though the underlying mechanisms remain elusive. In this study, we demonstrated that exposure to side-stream smoking inhibited mucosal inflammation, improved gut tight junction protein expression, and altered gut microflora composition in mice, which could partially explain the preventive effects of smoking on ulcerative colitis.

**Innovations and breakthroughs**

Recent epidemiologic studies have highlighted the preventive effect of smoking on ulcerative colitis. This is the first study to report that side-stream smoking has anti-inflammatory effect on gut mucosal, improving gut tight junction protein expression and altering gut microflora composition.

**Applications**

By understanding how side-stream smoking affects gut mucosal immune response and tight junction protein expression, the authors can develop alternative strategies to reduce the risk of ulcerative colitis and possibly other inflammatory bowel diseases without the harmful effects of smoking.

**Terminology**

Inflammatory bowel diseases are characterized by chronic inflammation in the intestine. Side-stream smoking, mimicking secondhand smoking, has anti-inflammatory effect, which may be responsible for its beneficial effects against ulcerative colitis.

**Peer review**

The authors address the observation that passive smoking decreases inflammatory response in large intestine. The authors are to be commended for excel-
lent work in performing a very important and informative study. The experimental methods are well summarized and explained. The statistics are appropriate for this study.

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