Probiotics Ameliorate Colon Epithelial Injury Induced by Ambient Ultrafine Particles Exposure

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Diesel exhaust particles (DEPs) are common airborne ultrafine particles (UFPs); however, few studies have examined their effects on the gastrointestinal tract. To investigate the interaction of gut microbiota and DEPs-induced colonic injury, adult C57BL/6 mice are kept in whole-body inhalation chambers and exposed to filtered room air (FRA) or DEPs (300 μg m^{-3}) 1 h per day for 28 consecutive days. DEPs exposure results in colon epithelial injury with inflammatory cell infiltration and mucus depletion. Abundance of *Lactobacillus* in murine feces is transiently increased following 7-day DEPs exposure and then decreased until the end of 28-day exposure. A reduction of the colonic mucus layer thickness is observed in mice receiving gut microbiota from DEPs-exposed mice. Mechanistically, RNA-sequencing suggests disruption of the nitrogen metabolism pathway in DEPs-exposed NCM460 cells. Upregulation of carbonic anhydrase 9 (CA9) expression levels is observed in epithelia following DEPs exposure both in vivo and in vitro. Oral administration of probiotics protects the mice against DEPS-induced colon epithelial injury. The results strongly suggest the involvement of gut microbiota in response to DEPs exposure and subsequently epithelial injury in vivo. Supplementation with probiotic may be a potential way to protect against UFPs-induced colon epithelial injury.

1. Introduction

Particulate matter (PM) pollution has been associated with pulmonary and extra-pulmonary injuries for decades. Recent studies have reported effects on the gastrointestinal tract[1,2] and with an increased risk for appendicitis, digestive tract cancers, and inflammatory bowel disease (IBD).[3] Intestinal exposure to PM occurs via mucocilliary clearance from the lungs and ingestion of contaminated food and water.[4] The physiochemical characteristics and biological effects of airborne ultrafine particles (UFPs) are distinguishable from the lager airborne particles (such as fine particulate matter ($PM_{2.5}$) and PM_{10}).[5,6] As a major component of traffic pollution, diesel exhaust particles (DEPs) are airborne ultrafine particles (UFPs), with an aerodynamic diameter less than 100 nm.[7] DEPs are comprised of organic and inorganic

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substances and have been considered a major contributor to the adverse health effects posed by ambient air pollutants.[8]

The gut microbiota is critical for human health[9,10] and maintains intestinal epithelial homeostasis offering a level of protection against gut injury.[11,12] Environmental stress can alter the gut microbiota and subsequently influence the host stress response.[13] Animal models have demonstrated adverse intestinal effects following oral administration or pulmonary inhalation of PM. For example, in a low-density lipoprotein receptor-null ($Ldlr^{-/-}$) mouse, oral ingestion of PM promoted lipid metabolism and inflammatory responses in small intestine and reduced the diversity of gut microbiota[2,14] and altered its composition.[15,16] Dysbiosis of the gut microbiota has been reported following PM inhalation[15,16] and associated with elevations in pro-inflammatory cytokines in the murine colon.[17] Specific for DEPs, intragastric administration to adult rats resulted in DNA damage and an oxidative stress response in colon epithelial cells.[18] Ingestion of DEPs by mice promotes lipid metabolism, villus shortening, and inflammatory response in the small intestine.[2] Above studies only reported the intestinal effects of DEPs via ingestion. So far, the effects of inhalational DEPs on colorectum, as well as gut microbiota, remain unknown.

In the current study, we examined the ability of inhaled DEPs to damage colonic epithelia and alter gut microbiota composition, as well as the potential protection of probiotics against DEPs-induced colonic injury.

2. Results

2.1. Experimental Design

In the present study, we hypothesized that dysbiosis of gut microbiota was involved in the DEPs inhalation-induced colon epithelial injury. We firstly observed the phenotype of epithelial injury and the alteration of gut microbiota composition in mice following DEPs inhalation; further, fecal microbiota transplant (FMT) suggested association between gut microbiota and epithelial injury. Secondly, an in vitro co-culture model of bacteria and colon epithelial cells was used for RNA-seq analysis, exploring the effects of gut bacteria on epithelia. Next, the expression levels of key molecules associated with DEPs exposure, as well as gut bacteria, were identified both in vitro and in vivo. Probiotics supplement to mice was used to observe the potential rescue against DEPs-induced colon mucus depletion.

2.2. Inhalation of DEPs Led to Colon Epithelial Injury

The mice were exposed to filtered room air (FRA) or DEPs for 28 consecutive days. Body weight was not found to be significantly different between DEPs- or FRA-exposed groups at each time point. Next, we examined the histopathological alterations in murine colonic tissues including H&E, Alcian blue, and periodic acid–Schiff (PAS) staining on the Swiss-roll sections (**Figure 1**A). At day 7 of the exposure, colonic tissues from DEPs-treated mice showed significant signs of epithelial injury but without dramatic infiltration of inflammatory cells as compared with FRA-treated control mice (Figure 1B,C). Similar epithelial injury was observed in murine colonic tissues following 14- or 21-day DEPs exposure (Figure S1, Supporting Information). The epithelial injury could be observed at proximal, middle, or distal colon sites, and no differences have been observed according to these three sites (data not shown). Until day 28 of the exposure, inflammatory infiltration was observed in DEPs-exposed group and the epithelial injury scores went much higher as compared with FRA-treated control mice (Figure 1B,D). Neither the interaction effects between DEPs exposure and sex nor the sexual difference following DEPs exposure were identified (Figure 1C,D). Additionally, we fixed the whole colonic tissues containing feces in Carnoy's fluid and performed Alcian blue and PAS staining (Figure 1E) to examine the thickness of mucus layer. Both acidic and neutral mucus was damaged by the inhalation of DEPs, which was seen as early as the 7th day of exposure (Figure 1F,G). The loss of acidic and neutral mucus lasted throughout the experimental period by showing reduction of the thickness of mucus layer (Figure 1F,H). Consistent with the pathological alterations, neither the interaction effects between DEPs exposure and sex nor the sexual difference following DEPs exposure was identified (Figure 1G,H). Thus, inhalation of DEPs damages colonic epithelium with loss of mucus and infiltration of inflammatory cells, in which loss of mucus occurred prior to inflammatory infiltration.

2.3. Inhalation of DEPs Led to Gut Microbiota Dysbiosis

Intestinal mucus is secreted by intestinal goblet cells and the outer layer of colorectum mucus contains intestinal bacteria.[19] Due to the reduction of mucus layer in the murine colons following 7-day DEPs exposure, it is reasonable to explore whether the dysbiosis of gut microbiota occurs synchronously. We thus performed 16s rDNA sequencing to examine the alteration of fecal microbiota using stools collected on the 7th day of exposure. Non-metric multidimensional scaling analysis showed that inhalation of DEPs clearly altered the composition of gut microbiota with the clusters of DEPs group distributed differently from that of FRA group (**Figure 2**A). In depth analysis of data revealed that *Lactobacillus* was increased in the gut microbiota of DEPs-treated mice (Figure 2B,C). Next, we measured the abundance of *Lactobacillus* in the stool samples, which were taken weekly post exposure, from the mice exposed to either DEPs or FRA by qRT-PCR (Figure 2D). We found that *Lactobacillus* was transiently upregulated by the inhalation of DEPs for the 1st week but downregulated from the 2nd week post exposure and the decrease of *Lactobacillus* lasted throughout the rest of the experiment. There is neither interaction effects between sex and DEPs exposure nor the sexual difference identified of *Lactobacillus* abundance (Figure 2D,E). The results mentioned above suggested the dysbiosis of gut microbiota induced by DEPs inhalation. We hypothesized that *Lactobacillus* play a protective role in response to DEPs exposure, and the transient upregulation of gut *Lactobacillus* is a stress reaction to DEPs inhalation. However, the association between gut microbiota dysbiosis and colon epithelial injury remains unclear.

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relative abundance of 9 bacterial taxa and decreased the relative abundance of 15 bacterial taxa in murine fecal samples.[16] Increases in *Lachnospiracea* were observed by both Mutlu et al.[15] and Wang et al.[16] In our study, mice were exposed to DEPs and only the elevated abundance of *Lactobacillus* was identified following 7-day exposure. All the three studies used the C57BL/6 murine model and it seems that long-term PM exposure posed more severe dysbiosis in the gut microbiota. The difference among studies might be attributed to the period of exposure and the varied PM from different locations.

Gut microbiota has been widely studied and recognized to be involved in regulating the homeostasis of intestinal epithelium and contributing to pathophysiological processes.[10–12,21] Based upon the FMT experiment reported in the current study, we hypothesized that dysbiosis of gut microbiota is at least one of the causes for DEPs-induced colon epithelial injury. In line with our RNA-sequencing assay results, other studies strongly support the possibility that the gut microbiota is involved in metabolism and homeostasis of intestinal epithelium.[22] Here, we found that CA9 was consistently upregulated in the colon epithelium from DEPs-treated mice across the exposure period. Carbonic anhydrases (CA) function as enzymes catalyzing the reversible hydration of carbon dioxide into bicarbonate and protons,[23] among which CA9 is controlled via the hypoxia-inducible factor (HIF).[24] Therefore, the increased expression of CA9 observed in the present study suggests potential alteration of intestinal microenvironment, such as hypoxia which may have been induced by DEPs. Furthermore, several studies focusing on increased intestinal CA9 expression suggested its association with the development of colorectal cancers,[25,26] implicating the long-term effects of consistent elevated CA9 expression.

We found that *Lactobacillus* abundance increased during the early stage of exposure to DEPs. However, more prolonged DEPs exposure reduced the abundance of *Lactobacillus* in the gut microbiota, which may have contributed to damage to the colonic epithelium. We hypothesize that an increased abundance of *Lactobacillus* may serve to protect the colonic epithelium during the early period of exposure to DEPs. *Lactobacillus* is one of the commonly used probiotics in commercial products.[27] Probiotics are increasingly used to prevent or treat a variety of intestinal diseases, including inflammatory bowel disease (IBD), acute infectious diarrhea, and antibiotic-associated diarrhea.[28] Here, we found that *Lactobacillus* administration effectively suppressed CA9 expression, protected against mucus depletion in murine colonic tissues following DEPs exposure. Consistent with our results, oral administration of probiotic bacteria could restore epithelial barrier integrity, colon physiologic function, and reduce mucosal inflammatory cytokine secretion.[29] Therefore, our results suggest that supplementation of the diet with probiotics may provide some protection against DEPs-related colonic injury. This possibility may have public-health implications since it is not practical or economically feasible to eliminate DEPs exposure in the environment completely.

One limitation should be noted that 16s rDNA analysis also suggested several decreased bacterial taxa in gut microbiota following DEPs exposure; however, the potential function of these bacteria require further investigations.

4. Conclusion

In summary, our results provide evidence for the involvement of the gut microbiota in colon epithelial injury induced by inhalation to DEPs. Dysbiosis of gut microbiota triggered mucus depletion and subsequent epithelial damage, as well as inflammatory infiltration. Thus, manipulation of gut microbiota, such as treatment with probiotics, may potentially be useful in preventing or protecting against colonic injury caused by exposure to DEPs.

5. Experimental Section

Animal Husbandry: A total of 140 male and 84 female 8-week old C57BL/6 mice (20–22 g) were purchased from SLRC Laboratory Animal Co., Ltd., (China) and housed in polycarbonate cages (4–6 mice per cage by sex) with corn cob bedding in a specific pathogen-free (SPF) animal facility under a 12 h light/dark cycle, at a room temperature of 22.5 °C. Mice received rodent chow (Jiangsu Xietong Pharmaceutical Bio-engineering Co., Ltd., China) sterilized by cobalt (Co) 60 radiation and autoclaved water ad libitum. Mice were allowed a 3-day acclimation to the facility. All animals were treated humanely, with regard for alleviation of suffering, and experimental procedure was approved by the Committee on Animal Use and Care of Southeast University, China.

Inhalational DEPs Exposure: A total of 112 C57BL/6 mice were randomly assigned to FRA (male $n = 28$ and female $n = 28$) or DEPs (male $n = 28$ and female $n = 28$) groups. The mice in DEPs group were exposed to DEPs with a mean concentration of 300 μg m^{-3}.[30] The mice in control group received FRA at the same flow rate as the DEPs group. The mice were exposed to FRA or DEPs for 1 h per day (from 9:00 a.m. to 10:00 a.m.) during the experimental period for 28 days. Briefly, the experiment was conducted as follows. Mice in their original cages were kept in stainless-steel Hinners-type whole-body inhalation chambers that were outfitted with air quality monitoring equipment and a dust aerosol generator (Beijing HuiRongHe Technology Co., Ltd., China). The concentration of dry DEPs (the Standard Reference Material 2975, National Institute of Standards and Technology, NIST, USA) aerosol was adjusted by the rotation speed of the rotary brush outfitted on the dust aerosol generator. The mass concentrations of DEPs aerosol in the whole-body chamber were measured by a real-time light scattering dust monitor (CEL-712 Microdust Pro, CASELLA CEL Inc., USA). At the termination of each daily exposure, mice were removed from the chambers and maintained in their home cage.

Histopathological Analysis: Mice (seven males and seven females from each group) were randomly selected, weighted, euthanized under ether anesthesia following 7, 14, 21, or 28 days of exposure. Necropsies were performed and colonic tissues harvested. Four colonic tissues/sex/group were immersion fixed in 4% paraformaldehyde, and processed for paraffin embedding. Sections were rehydrated and stained by hematoxylin and eosin (H&E), Alcian blue, or Periodic Acid-Schiff stain (PAS) for epithelial injury and inflammatory infiltration analysis. Three colorectal tissues/sex/group were immersion fixed in Carnoy's fluid to observe the integrity of colonic mucus layer by Alcian blue and PAS staining. Sections were scanned using Panoramic SCAN (3DHISTECH, Hungary) to obtain a whole slide image.

Each section was scored across the entire section for epithelial injury and inflammatory infiltration. Epithelial injury scores were calculated by summarizing the scores for gland mucodepletion (0, none; 1, mild; 2, moderate; 3, severe) and scores of tissue damage (0, no mucosal damage; 1, discrete epithelial lesions; 2, surface mucosal erosion or focal ulceration; 3, extensive mucosal damage and extension into deeper structures of the bowel wall). Inflammatory infiltration score was calculated by summarizing infiltration score (0, no inflammatory cells; 1, infiltration around crypt bases; 2, infiltration of muscularis mucosa; 3, infiltration of submucosa) and the percent area of each section (0, no involvement; 1, ≤25%; 2, ≤50%; 3, ≤75%; 4, ≤100%).

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