Protective action of NADPH oxidase inhibitors and role of NADPH oxidase in pathogenesis of colon inflammation in mice

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Abstract

AIM: To investigate the role of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in colon epithelial cells in the pathogenesis of acute and chronic colon inflammation in a mouse model of dextran sulphate sodium (DSS)-induced colitis.

METHODS: Balb/c mice were divided into three groups: 8 mice with acute DSS-induced colitis (3.5% DSS solution; 7 d), 8 mice with chronic DSS-induced colitis (3.5% DSS solution for 5 d + water for 6 d; 4 cycles; total: 44 d) and 12 mice without DSS supplementation as a control group. Primary colonic epithelial cells were isolated using chelation method. The cells were cultivated in the presence of mediators (lipopolysaccharide (LPS), apocynin or diphenyleneiodonium). Viability of cells was assessed by fluorescent microscopy. Production of reactive oxygen species (ROS) by the cells was measured fluorometrically using Amplex Red. Production of tumour necrosis factor-alpha (TNF-α) by the colonic epithelial cells was analysed by ELISA. Nox1 gene expression was assessed by real-time PCR.

RESULTS: Our study showed that TNF-α level was increased in unstimulated primary colonic cells both in the acute and chronic colitis groups, whereas decreased viability, increased ROS production, and expression of Nox1 was characteristic only for chronic DSS colitis mice when compared to the controls. The stimulation by LPS increased ROS generation via NADPH oxidase and decreased cell viability in mice with acute colitis. Treatment with NADPH oxidase inhibitors increased cell viability and decreased the levels of ROS and TNF-α in the LPS-treated cells isolated from mice of both acute and chronic colitis groups.

CONCLUSION: Our study revealed the importance of NADPH oxidase in the pathogenesis of both acute and chronic inflammation of the colon.

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Key words: Apocynin; Diphenyleneiodonium; Dextran sulphate sodium-induced colitis; Inflammatory bowel disease, Nicotinamide adenine dinucleotide phosphate oxidase; Reactive oxygen species; Tumour necrosis factor-α
Core tip: Reactive oxygen species (ROS)-induced oxidative stress is one of the most important etiologic factors involved in the inflammation. The key producers of ROS in cells are nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase enzymes. Several studies have shown that epithelial NAPDH oxidase might be responsible for normal immune response to antigens in the gut. On the other hand, little is known about the molecular pathways controlling ROS production via NAPDH oxidase in primary intestinal epithelial cells during inflammation. The aim of this study was to investigate the role of NAPDH oxidase in colon epithelial cells in the pathogenesis of acute and chronic colon inflammation using a mouse model of dextran sulphate sodium-induced colitis. The results of our study revealed the importance of NAPDH oxidase in the pathogenesis of colon inflammation.

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) that affects the intestinal mucosa[1]. The pathogenesis of UC seems to involve primary defects in one or more elements responsible for the recognition of bacteria and normal immune response to antigens in the gut[2,3]. Previous studies indicated the importance of reactive oxygen species (ROS)-induced oxidative stress in the development of IBD. The key producers of ROS in phagocytic and non-phagocytic cells are NAPDH oxidase enzymes[4,5]. NAPDH oxidase-derived ROS act as intracellular messengers for a variety of cellular receptor signal transduction pathways, and play pivotal roles in various biological activities, including host defence, cell growth and differentiation, stimulation of pro-inflammatory genes, and cell death[6,8]. The epithelial NAPDH oxidase homologs (Nox1 and DUOX2) generate a higher level of ROS in the colon compared to phagocytic NAPDH oxidase (Nox2)[9]. Nox1, the so-called "colon NADPH oxidase", is highly expressed in the colon, particularly in colon epithelial cells[10]. This enzyme comes into close contact with normal and pathogenic bacteria and may play an important role in local innate immune and inflammatory responses in the gut[9,10,11]. Several studies have shown that bacterial products and pro-inflammatory cytokines such as interleukin-18 (IL-18), interferon gamma and tumour necrosis factor-alpha (TNF-α) can stimulate the NAPDH oxidase expression and ROS production in intestinal epithelial cell cultures in vitro[10,11]. Anti-inflammatory cytokines interleukin-10 (IL-10) and transforming growth factor-beta effectively block the stimulatory actions of pro-inflammatory cytokines and decrease NAPDH oxidase activity in colon cancer cell line[10,11]. However, molecular pathways controlling ROS production via NOX enzymes in primary intestinal epithelial cells during acute and chronic inflammation are poorly understood.

The aim of this study was to investigate the role of NAPDH oxidase in colon epithelial cells in the acute and chronic colon inflammation using mice with dextran sulphate sodium (DSS)-induced colitis.

MATERIALS AND METHODS

Animals

Male Balb/c mice were used for the experiments (Lithuanian University of Health Sciences, Veterinary Academy, vivarium, Lithuania). All mice were 6-8-wk-old and had an approximate weight of 16-20 g at the beginning of the experiment. Mice were housed in individual plastic cages (1 mouse per cage) in a 12-h light/dark cycle at 22 °C room temperature and were provided with food and water ad libitum. Procedures involving animal care were conducted conforming to national and international laws and policies. The experimental design was approved by the Lithuanian Animal Ethics Committee (Protocol No. 0201).

Induction of colitis with DSS

Colon inflammation in Balb/c mice was induced by oral administration of 3.5% DSS dissolved in the distilled drinking water and supplied ad libitum (molecular mass 40 kDa, TdB Consultancy, Uppsala, Sweden). We used a protocol that was established by Wirtz et al[12], which was slightly modified as follows: animals were divided into three study groups: 8 mice with acute DSS-induced colitis (mice were given 3.5% DSS in the drinking water over 7 d; 1 cycle; total number of days: 7), 8 mice with chronic DSS-induced colitis (mice were given 3.5% DSS in the drinking water over 5 d and water for 6 d; this cycle was repeated 4 times; total number of days: 44), and 12 mice as a control group without DSS supplementation.

Evaluation of colonic inflammation

Assessment of clinical parameters: Clinical parameters recorded in the experiments with Balb/c mice were colon length (cm), colon weight (mg, without faeces), spleen weight (mg), body weight (g), mortality, diarrhoea (assessment of faeces was performed using the Bristol scale) and rectal bleeding (seen by ocular inspection)[13]. Bristol scale is designed to classify faeces into seven groups according to faeces consistency in points from 1 (very hard) to 7 (entirely liquid)[14]. The clinical and morphological parameters of the animals were expressed as a mean of the group.

Assessment of histological score in mice: All chemi-
Isolation and cultivation of primary mouse colonic epithelial cells

Large intestine was removed and placed in PBS for washing procedure. Subsequently, the intestine was cut into 1-mm fragments and transferred in PBS solution containing 50 IU/mL penicillin, 50 μg/mL streptomycin, and 0.5 mg/mL gentamycin. The primary mouse colonic epithelial cells were isolated using chelation method as described by Meijsen et al, but slightly modified as follows. The intestine fragments were incubated in 2.5 mmol/L EDTA dissolved in PBS for 40-45 min at room temperature to liberate the single epithelial cells from the lamina propria. The isolated epithelial cells were suspended in DMEM supplemented with 15% fetal calf serum, 10 μmol/L of LPS, or 20 μg/mL of lipopolysaccharide (LPS), 1 mmol/L of apocynin, 20 μg/mL of DPI for 30 min at 37℃ and immediately fixed with 10% neutral formalin for 4 h at room temperature for paraffin embedding. Serial 3-μm sections were cut for each tract and stained with haematoxylin and eosin for histological examination. Images of tissue were analyzed using an OLYMPUS IX71 microscope with Q IMAGING EX1 aqua camera (Tokyo, Japan). Histological examination was performed using the method suggested by Hausmann et al.

Assessment of cell viability

The viability of cultured mouse colonic epithelial cells was assessed by propidium iodide (PI, 7 μmol/L) and Hoechst 33342 (4 μg/mL) staining using an OLYMPUS IX71S1F-3 fluorescence microscope (Tokyo, Japan). PI-negative cells with weak Hoechst-staining were considered to be viable, whereas cells showing nuclear shrinkage or fragmentation and intensive Hoechst staining but still lacking PI staining were classified as necrotic. Mouse colonic epithelial cells were counted in at least 5 microscopic fields per well (three wells per treatment). Data are expressed as percentage of viable, necrotic or apoptotic cells of the total number of cells per field.

Assessment of TNF-α concentration

Concentration of TNF-α was assessed in the supernatants of primary mouse epithelial cell cultures using a commercially available two-site ELISA kit (Invitrogen, Carlsbad, CA). The lowest limit of sensitivity of the test system for TNF-α was 3 pg/mL. The optical densities at 450 nm and at a correction wavelength of 490 nm were measured on an ELISA microplate reader (MRX microplate reader, Dynex Technologies, Denkendorf, Germany).

Assessment of gene expression in colonic epithelial cells by real-time PCR

Isolated mouse epithelial cells were stabilised with RNAlater® solution and kept in -80℃ until further analysis. Total RNA was isolated using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Vilnius, Lithuania). Purified RNA was re-suspended in RNase-free water and stored at -80℃. RNA purity and integrity were assessed using a spectrophotometer (Nanodrop Technologies, Wilmington, DE, United States). cDNA was synthesised from total RNA (200 ng) using First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania). Reaction for the real-time PCR was performed using predesigned TaqMan® Gene Expression Assays (Gene expression assays IDs: Nool - Mm00549170_m1, Aeth - Mm00607939_s1, Ptprc - Mm01293577_m1; Cad3e...
Table 1 Assessment of clinical symptoms and morphological alterations after dextran sulphate sodium administration in Balb/c mice

| Group         | n | Length of colon (cm) | Colon weight (mg) | Weight of colon/length of colon | Spleen weight (mg) | Body weight (g) | Assessment of faeces | Rectal bleeding | Mortality |
|---------------|---|----------------------|-------------------|-------------------------------|-------------------|----------------|---------------------|----------------|-----------|
| Control       | 12 | 7.8 ± 0.78          | 337.6 ± 69.04     | 44.26 ± 7.11                  | 97.1 ± 31.35      | 21.9 ± 3.89     | 2.5 ± 0.52          | 0/12           | 0/12      |
| Acute colitis | 8  | 5.9 ± 0.97          | 321.9 ± 55.29     | 55.03 ± 6.48                  | 197.9 ± 93.58     | 17.0 ± 2.30      | 5.0 ± 0.85          | 5/8            | 0/8       |
| Chronic colitis | 8 | 7.2 ± 0.27          | 354.2 ± 69.29     | 49.39 ± 6.28                  | 119.0 ± 20.74     | 18.8 ± 1.06      | 4.2 ± 0.45          | 0/8            | 0/8       |

Colon inflammation was induced by the administration of 3.5% DSS solution in Balb/c mice. Assessment of faeces was performed using the Bristol scale (points 1 to 7). Rectal bleeding - number of mice with rectal bleeding (percentages)/total number of mice (percentages). Mortality - number of dead mice (percentages)/total number of mice (percentages). *P < 0.05, acute DSS-induced colitis groups vs control; †P < 0.05, chronic DSS-induced colitis groups vs control; ‡P < 0.05, acute DSS-induced colitis vs chronic DSS-induced colitis groups. Results are presented as mean ± SE.

- Mm00599684_g1; Cd68 - Mm03047340_m1; V’il - Mm00457074_m1 and TaqMan® Universal PCR Master Mix, according to the manufacturer’s instructions (Life Technologies, Carlsbad, United States). Actb was used as the endogenous control gene. The experiments for each sample were performed in triplicate. Expression in the mouse epithelial cells was determined using ABI Fast 7500 System (Life Technologies, Carlsbad, CA, United States). The PCR conditions were 1 cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The changes of gene expression was analysed using the 2^ΔCT method.

**Statistical analysis**

Statistical analyses were performed using the SPSS statistical package (version 16.0; Chicago, IL). Data in text and figures are presented as mean ± standard error. Statistical analyses were performed using one-way ANOVA. The least significant difference test was used as a post hoc test. A P-value < 0.05 was considered statistically significant.

**RESULTS**

**Assessment of the clinical symptoms of colitis mice**

Oral administration of 3.5% DSS solution for 7 d induced severe acute colitis in mice with a significant reduction in the body weight, diarrhoea, rectal bleeding, decreased colon length, increased spleen weight when compared to control mice (Table 1). Administration of 3.5% DSS for 44 d induced less severe damage, however, a significant body weight loss and liquefaction of faeces were observed compared to the control mice (Table 1). The animals with acute colitis had more severe symptoms in comparison to chronic colitis mice, including pronounced weight loss, rectal bleeding, decrease in colon length, and increase in spleen weight. The mice in the control group did not show any clinical symptoms of the disease.

**Histological assessment of colon inflammation in mice**

In mice with acute colitis we observed major epithelium damage with loss of crypts in large areas (3.8 ± 0.42, P = 0.01) and inflammatory cell infiltration of the lamina submucosa (3.9 ± 0.35, P = 0.01) (Figure 1B). In mice with chronic colitis, loss of goblet cells in large areas (2.9 ± 0.19, P = 0.02) and inflammatory cells infiltration of the lamina muscularis mucosae (2.2 ± 0.30, P = 0.02) were observed (Figure 1C). Control mice had no histological alterations in the colon tissue (Figure 1A).

**Assessment of purity of isolated epithelial cell culture**

The purity of the primary colonic epithelial cell culture was assessed by detecting the expression of gene markers (Ptprc - a hematopoietic cell marker; Cd3e - a T cell marker; Cd68 - a monocyte/macrophage marker; V’il - an epithelial cell marker). As shown in Figure 2, the expression levels of Ptprc, Cd3e, and Cd68 gene markers, specific for inflammatory cells, were minor compared to the expression of epithelial cell marker V’il in primary colonic epithelial cells. Moreover, expression levels of these genes were identical in cell cultures obtained from all three experimental groups. These findings indicate that inflammatory cells did not affect ROS, TNF-α production and Nox1 expression in our cell culture.

**Assessment of viability of mouse colonic epithelial cells**

The viability of cells isolated from chronic colitis mice after 24 h was significantly lower and number of necrotic cells was higher than those in the control group, whereas no significant difference between acute colitis and control groups was observed (Figure 3A, B and C). Treatment of cells with NADPH oxidase inhibitor apocynin substantially increased the viability of cells (to 68%) and reduced necrosis (to 30%) in mice with chronic colitis, whereas apocynin had no significant effect on cell survival in the control and acute colitis groups (Figure 3C). Stimulation of colonic epithelial cells with LPS significantly reduced the viability of the cells in the control (to 50%) and both acute and chronic colitis groups (to 47% and to 38%, respectively) by increasing the number of necrotic cells (Figure 3A, B and C). However, treatment with apocynin prevented the inhibitory effect of LPS on colonic epithelial cell viability in all groups of mice. Cell viability was significantly increased to 60% in the control group, to 64% in the acute colitis and to 55% in...
chronic colitis groups and number of necrotic cells was significantly decreased (36%, 32% and 40%, respectively, Figure 3A, B and C). The percentage of apoptotic cells was minor (3%-8%) in all study groups.

Assessment of hydrogen peroxide production in cells

The level of extracellular hydrogen peroxide production was significantly higher in chronic colitis mouse cells than in the control group (Figure 4A). Similar results were obtained when hydrogen peroxide production was measured directly in the colonic biopsies, i.e., hydrogen peroxide production was higher in the biopsies of both colitis groups when compared to controls (Figure 4B).

NADPH oxidase inhibitor DPI decreased the level of ROS production in the chronic colitis group when compared to the control by 1.5 times (Figure 4A). Treatment of cells with bacterial endotoxin LPS significantly increased the production of hydrogen peroxide in both colitis groups (1.5-fold higher in acute colitis group and 1.3-fold higher in chronic colitis group). The addition of DPI to LPS-treated colonic epithelial cells significantly decreased the level of ROS production in all experimental colitis groups. In the acute colitis group, ROS level was approximately 1.6-fold lower compared to the LPS-treated cells. Similar results were obtained in the chronic colitis group, where hydrogen peroxide production decreased approximately 1.5-fold (Figure 4A).

Assessment of TNF-α concentration in colonic epithelial cells

We investigated the influence of NADPH oxidase on the production of pro-inflammatory cytokine TNF-α.
As shown in Figure 5, the level of TNF-α was significantly increased in both acute and chronic colitis groups compared to the control (4.5 and 3.6-fold, respectively). In chronic colitis mice treatment with LPS nearly doubled the levels of TNF-α production. Treatment with NADPH oxidase inhibitor apocynin prevented the stimulating effect of LPS on TNF-α production in primary colonic cells in both acute and chronic colitis groups (1.5 and 1.6-fold, respectively, Figure 5).

Expression of Nox1 in colonic epithelial cells
We investigated the expression levels of Nox1 in the colonic epithelial cells of mice using real-time PCR analysis. As demonstrated in Figure 6, the expression level of Nox1 in the epithelial cells during chronic colitis was approximately 6.4-fold higher when compared to controls. Expression level of Nox1 did not differ significantly in the epithelial cells isolated from mice with acute colitis and the control group.

DISCUSSION
Several studies have shown that epithelial NADPH oxidase mediated formation of ROS is involved in inflammatory responses and host defence system at mucosal surfaces19,20,21. Increased production of ROS via NADPH oxidase has been implicated in tissue damage observed in chronic inflammatory disorders, such as IBD1,21,22. In this study, using an experimental colitis mouse model, we examined the role of NADPH oxidase in colon epithelial cells in the acute and chronic colon inflammation.

The results of this study revealed that expression level of Nox1 is increased in the colon epithelium of mice with chronic colitis. Similar findings were observed in the human colon epithelium of patients with ulcerative colitis21. Unstimulated cells of chronic colitis mice had decreased viability and increased ROS production via NADPH oxidase and TNF-α concentration when compared to the control group. This observation suggests that NADPH oxidase is an active player in chronic inflammatory response in the primary colonic epithelial cells. Increased activity of NADPH oxidase triggers unbalanced inflammation cascade and non-phagocytic cell damage, and can act as an oncogenic factor which increases the risk of intestinal cancer21,22. Furthermore, overproduction of ROS may induce chronic inflammation5 and damage of the colon tissue5,23.

Interestingly, we did not find significant differences in Nox1 expression, cell viability or ROS synthesis in the colonic epithelium of mice with acute colitis when compared to the control mice. Nevertheless, TNF-α level was increased in acute colitis mice, indicating active inflam-
In order to eliminate the potential bias for our results, the purity of colonic epithelial cell culture was confirmed by specific cell markers Peyer, Cdc42, Cdc68 and Wil. The expression analyses of these cell markers revealed that colonic epithelial cells produced inflammation mediators themselves and generated inflammatory responses to antigens.

The inhibition of NADPH oxidase represents an attractive therapeutic strategy for the treatment of many chronic diseases. Apocynin has been widely used as a selective inhibitor of the complex NADPH oxidase in the experimental models of inflammation. Apocynin possesses very low toxicity and anti-inflammatory activity, i.e., reducing the level of inflammatory cytokines including TNF-α, protecting cells from damage induced by bacterial products and decreasing damage in the colon tissue. In our study in both colitis groups, apocynin decreased necrosis and TNF-α production and increased cell viability in LPS-treated colonic epithelial cells. Similar findings were determined in the chronic colitis group, where apocynin substantially increased the viability of cells and reduced necrosis. We hypothesize that the protective mechanism of apocynin might be associated not only with anti-inflammatory action of this inhibitor but also with the decreased ROS generation via NADPH oxidase and reduced oxidative stress in cells.
Recently, this effect of apocynin was observed in the primary colonic epithelial cells of patients with ulcerative colitis. However, the effect of apocynin on colon epithelial regeneration needs to be investigated in the future.

The results of our study showed that epithelial NADPH oxidase is directly involved in chronic colon inflammation; however, stimulation by bacterial products is required for NADPH oxidase activation during acute colitis. In both cases, the molecular mechanism for activation of NADPH oxidase is similar. The signalling cascade for activation of NADPH oxidase in colonic epithelial cells might be associated with the toll-like receptor (TLR) pathway, where LPS strains potently stimulate ROS production by Nox1 through TLR4. Further studies should be designed to investigate the role and exact mechanism of LPS/TLR4/TFN-α/NOX signalling in the intestine epithelium in acute and chronic colitis. The detection of expression changes of other NADPH oxidase homologs and evaluation of superoxide in primary epithelial cells during colon inflammation should be conducted in novel studies.

In conclusion, our study revealed the importance of NADPH oxidase in pathogenesis of acute and chronic colon inflammation. Moreover, treatment with NADPH oxidase inhibitors had a protective impact against pro-inflammatory action of bacterial endotoxins in mouse colon epithelial cells during acute and chronic colitis.

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