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Chapter 8.

Acetylcholine protects against inflammatory cytokine induced epithelial barrier dysfunction

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Abstract

**Background:** Increased epithelial permeability during chronic inflammation is a perpetuating factor in inflammatory bowel disease. Pro-inflammatory cytokines (TNF-α and IL-1β) are shown to increase epithelial permeability. In animal models, vagus nerve-derived acetylcholine (Ach) ameliorates intestinal inflammation via activation of cholinergic receptors on innate inflammatory cells.

**Aim:** To investigate whether Ach confers protection to intestinal inflammation via modulation of chemokine release and maintenance of barrier function in human epithelial cells.

**Methods:** Monolayers of Caco-2 cells were incubated with cholinergic agonists and/or an inflammatory cytokine mix (TNF-α/IL-1β) and changes in IL-8 production, transepithelial electrical resistance (TEER), and transepithelial FITC-dextran flux were analysed in co-culture systems. Occludin and ZO-1 expression were analysed by immunoblot and immunofluorescence, respectively.

**Results:** Ach and muscarine, but not nicotine reduced IL-1β-induced NF-κB activity in Caco-2 cells. This effect was mediated via mAChRs, as muscarine and Ach were equally potent in reducing IL-8 while nicotine was not effective. In conjunction, IL1-β or TNF-α induced IL-8 production by Caco-2 cells was inhibited by Ach in a dose-dependent fashion. Ach enhanced epithelial permeability under steady state conditions. On the other hand, Caco-2 cells exposed to cytokines (IL-1β/TNF-α) for 72 hours showed a reduced barrier function reflected in a reduced TEER, increased dextran flux and loss of ZO-1 expression, and this impaired barrier integrity was counteracted by Ach and muscarine, but not nicotine.

**Conclusion:** Ach protects epithelial cells from the detrimental effects of IL-1β and TNF-α on the integrity of the intestinal epithelial barrier via activation of mAChRs.
Introduction

The intestinal epithelial layer has developed complex barrier mechanisms to prevent unrestricted access of luminal contents in the lamina propria, while still allowing dendritic cell-mediated surveillance of luminal antigens. Barrier proteins involved in this process include adherens junctions, desmosomes, gap junctions and tight junctions (TJs). TJs or zonula occludens are the most apical components of these intercellular junctions. The main functions of the TJs are to prevent diffusion of membrane proteins and lipids between basolateral and apical membranes so that cell polarity is preserved (fence function) and to function as a selective barrier to paracellular transport (barrier function). It has been demonstrated that intestinal epithelial barrier function is impaired in a range of inflammatory disease states such as inflammatory bowel diseases (IBD), irritable bowel syndrome, post-operative ileus, celiac disease, food allergy, asthma and rheumatoid arthritis \(^1\)-\(^4\). The impaired epithelial barrier function is likely to contribute to the severity of chronic inflammation.

TJs are composed of a complex of membrane proteins, including occludin, several members of the claudin family and junctional adhesion molecule-1 \(^5\)-\(^12\) and a family of zonula occludens (ZO) proteins that connect the membrane part to the actin filaments. It has been shown that pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-\(\alpha\), interleukin (IL)-1\(\beta\), interferon (IFN)-\(\gamma\) and IL-8 increase intestinal permeability by rearrangement and internalisation of TJ proteins, probably mediated by a rapid activation of transcription factor NF-\(\kappa\)B \(^13\)-\(^19\). Bacterial recognition occurs on enterocytes and colonocytes via their apical expression of a range of Toll-like receptors (TLRs), but epithelial cells generally show enhanced barrier integrity on TLR ligation, via enhanced TJ expression and rearrangement \(^20,21\).

Intestinal barrier function is highly regulated by neuronal factors, such as enteric glial cells (EGCs) \(^22\). In addition, recently it has been shown that enhanced vagus nerve motor output, either stimulated pharmacologically, electrically, or via
high fat nutrition \(^{23}\), serves a protective effect on barrier function under conditions of intestinal inflammation. Cholinergic activity however acts as a double-edged sword: although protective in inflamed conditions, under healthy conditions vagus nerve efferent activation was shown to enhance bacterial translocation and paracellular permeability in the gut \(^{24-26}\) and Ach increases transcellular transport via muscarinic Ach receptor (mAchR) activation \(^{27}\). These data seem contradictory since an increased intestinal epithelial barrier leads to an increased exposure of antigens to the mucosal compartment.

Thus, vagal- or cholinergic activity may favour an enhanced immune surveillance under healthy conditions, while maintaining barrier function in disease. Hence we investigated molecular mechanisms of functional changes of cholinergic modulation of the barrier function and focussed on healthy and inflamed conditions. In the human intestinal epithelial cell line Caco-2 we demonstrate that cholinergic agonists reduce TNF-\(\alpha\) or IL1-\(\beta\) induced NF-\(\kappa\)B activation and IL-8 production. This effect is mediated via mAchR, since only Ach and muscarine decreases TNF-\(\alpha\) and IL-1\(\beta\)-induced IL-8 production. Nicotine, which acts on nicotinic acetylcholine receptors (nAchRs), decreases only IL-8 production that is induced by low concentrations of IL-1\(\beta\). Moreover, while Ach and muscarine, reduce IL-1\(\beta\)-induced NF-\(\kappa\)B activation, nicotine fails to counteract NF-\(\kappa\)B activity. In cytokine stimulated epithelial cells, Ach protects against enhanced transcellular transport and distorted ZO-1 expression.
Materials and methods

Cell culture

Caco-2 cells (kindly provided by J. Plat, University of Maastricht, Maastricht, The Netherlands) were cultured in DMEM, 10% foetal calf’s serum (heat inactivated), 5% glutamine and 5% penicilline/streptavidine (all obtained from Gibco BRL, Breda, The Netherlands) in a 75 cm² flask. Adherent cultures passaged weekly at subconfluence after trypsinisation. The cells were maintained at 37°C in an atmosphere of 5% CO₂.

After culturing Caco-2 cells in a flask, they were cultured in 12 or 24-wells plates or transwells (Costar, Cambridge, MA, USA). Cells were used for further experiments between 14 and 21 days of confluence or when transepithelial electrical resistance (TEER) was constant over time. The TEER in transwells was measured with a Millipore-ERS metre (Millipore corp., Bedford, MA).

NF-κB reporter assay

Caco-2 cells that were stably transfected with an NF-κB luciferase reporter construct were cultured in 12-wells plate and the cells were washed with PBS followed by refreshing the medium approximately 2 to 3 hours before the experiment. At the start of the experiment, the medium was replaced by medium containing 25ng/ml IL-1β and 10, 100 or 1000 nM Ach (Sigma, St. Louis., MO). One well was kept blank to serve as a treatment control. Thereafter the cells were incubated at 37°C for 16 hours. Cells were washed with PBS and passive lysis buffer (Promega, Madison, WI) was added to the cells followed by scraping the attached cells from the dish. The cell lysates were stored at -80°C until further use.

Luciferase Assay Reagent (Promega) was dispensed into a luminometer plate well (Grenier Bio One ltd. Stonehouse, UK). The luminometer was programmed to perform a 2-second measurement delay followed by a 10-second
measurement read for luciferase activity (sensitivity 240). Cell lysate was added to the Luciferase Assay Reagent in the luminometer plate and was placed in the luminometer to measure luciferase activity.

**IL-8 measurement and isolation of protein**

Caco-2 cells were cultured in 24-wells plates and between 14 and 21 days after confluence, the cells were stimulated with 0, 100, 1000 or 10,000 nM Ach, nicotine or muscarine (all from Sigma) for 20 minutes. Thereafter, Ach, nicotine or muscarine were washed away with PBS and the medium was replaced by DMEM containing different concentrations of IL-1β (Miltenyi), TNF-α (Miltenyi) or no stimuli and in case of Ach stimulation 50nM of neostigmine (Sigma) may be added to protect intrinsic Ach from breakdown. After 3 hours, supernatant was collected to perform an IL-8 ELISA (Arcus Biologicals Srl, Modena, Italy) according to manufacturer’s manual.

Furthermore, membrane and cytosolic fractions were separated to perform western blots. Therefore, Caco-2 cells were scraped in PBS (4°C) and centrifuged at 1000 rpm for 5 minutes. Supernatant was removed and lysisbuffer was added to the cell pellet. The lysisbuffer was made of in 2mM EDTA (Sigma), 250mM mannitol (Sigma), 20mM Hepes/Tris pH 7.4 (Sigma) and protease inhibitors (Gibco). The cell pellet was left in lysisbuffer for 30 minutes on ice followed by douncing 30 times. This homogenate was centrifuged at 1000 rpm for 5 minutes. Supernatant was collected in an ultracentrifuge tube and centrifuged in an ultracentrifuge at 45000 rpm for 45 minutes. This collected supernatant contains the cytosolic fraction, whereas the pellet contains the membrane fraction. Membrane fractions were solved in PBS and to solve the fractions, a 21-gauge needle was used. All the protein fractions were stored at -20°C.
FITC-dextran permeability determination

Caco-2 cells were cultured on transwells for 72 hours in the presence or absence of a cytokine cocktail containing 50 ng/ml TNF-α, 10 ng/ml IL-1β and 50 ng/ml IFN-γ. Simultaneously, the cells were incubated with 0, 100 or 1000 nM Ach with or without 50nM neostigmine. One hour prior to flux experiment the media of all wells was refreshed with conditioned medium without phenol red (Gibco). At the mucosal compartment, 5μl FITC-dextran stock (25 mM in MEM-α, Sigma) was added. At t=30 and t=60 sample volumes of 100 μl were taken from the serosal compartment and collected in a white 96 wells plate (Corning, Lowell, MA) covered with aluminium foil. The samples were measured with the Fluoroskan Ascent FL (Thermo Scientific, Rockford, IL) (ex 492 em 518).

SDS-PAGE and western blotting

Protein contents of membrane and cytosol fractions were determined by the BCA assay (Pierce, Rockford, IL) according to the manufacturer’s recommendation. Equal amounts of protein of membrane and cytosol extracts were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and electroblotted to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked in 5% (w/v) non-fat dry milk (Fluka, St. Louis., MO) in TBS. Thereafter, the membrane was incubated with primary antibody against occludin (1:1000, rabbit anti occludin, Zymed Laboratories Inc., South San Francisco, California, USA), which was dissolved in 1% non-fat dry milk in TBST, at 4°C overnight. After washing with TBS, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit Ig antibody (Dako, Glostrup, Danmark) dissolved in 1% milk in TBST at room temperature for 1 hour. Finally, lumi-light substrate (Roche, Basel, Switzerland) was used to visualise the western blots. Pictures were made with the Lumi-Imager F1 (Roche). Equal protein loading was confirmed by western blots for β-actin.
Immunofluorescence

Caco-2 cells grown in transwells were fixed in 4% paraformaldehyde for 10 minutes. After pre-incubation with PBS, samples were blocked in 1% bovine serum albumine (BSA) (Sigma) and 0.1% triton (Sigma) in PBS for 15 minutes. Thereafter, the samples were incubated with polyclonal antibodies against ZO-1 (1:500, rabbit anti-ZO-1, Zymed Laboratories Inc., South San Francisco, California, USA) in 1% BSA and 0.1% triton in PBS for one hour at room temperature. After washing the samples with PBS, the samples were incubated with the secondary antibody (1:1000, goat anti-rabbit Alexa 546) in 1% BSA and 0.1% triton in PBS for 30 minutes. Finally, the samples were washed with PBS and covered with moviol, including DAPI.

Immunostaining and imaging. Confocal immune fluorescence on intestinal sections was performed as described earlier. The polyclonal antibody against LAMP-2 was kindly provided by S. Heinsbroek, University of Amsterdam.

Electrical vagal nerve stimulation and in vivo uptake

Electrical vagal nerve stimulation (VNS): Mice were anaesthetised by i.p. injection of a mixture of Fentanyl Citrate / Fluanisone (Hypnorm; Janssen, Beerse, Belgium) and Midazolam (Dormicum; Roche, Mijdrecht, The Netherlands). VNS was performed as described previously 29. In short: the right cervical vagal branch was prepared free from the carotid artery and ligated with 6-0 silk suture. The part distal from the ligation was attached to an electrode and 5V stimuli with a frequency of 5Hz, duration of 2ms10 were applied for 5 minutes. In sham mice the cervical skin was opened and left for 30 minutes covered by moist gauze. After a 30 minutes recovery period, the in vivo uptake assay was initiated as described below.

In vivo uptake: Surgical procedures were performed under sterile conditions. Mice underwent a laparotomy and an ileum segment 3-10 cm proximal from the caecum was opened and its lumen rinsed with pre-warmed (37°C) oxygenated Krebs buffer. The ileum segment was filled with 1-2 ml of buffer containing FITC-labelled...
E. faecium bacteria and clamped at both sides. After 30 minutes the clamped intestinal segment was removed, washed in PBS and processed for immunohistochemistry.

**Statistical analysis**

All data are expressed as the means ± the standard error of the mean (SEM) and were analysed using Graphpad prism 4 (Graphpad Prism v. 4 for Windows, GraphPad Software, San Diego, California USA). Differences between groups were analysed using the non-parametric Mann Whitney U test. All statistics were performed two-tailed and values of $p<0.05$ were considered statistically significant (* $p<0.05$; ** $p<0.01$, *** $p<0.001$).
Chapter 8

Results

Acetylcholine and muscarine inhibit NF-κB activity in Caco-2 cells

Since it has been shown that Ach inhibits the NF-κB pathway in immune cells\textsuperscript{30-36}, we wanted to investigate whether Ach is also able to inhibit NF-κB activity in intestinal epithelial cells. Therefore, an NF-κB reporter assay was performed to test whether Ach, nicotine and muscarine inhibit NF-κB activity of Caco-2 cells induced by IL-1β. It seems that Ach and muscarine inhibit NF-κB activity in a dose-dependent manner (see figure 1). This effect is less pronounced if Caco-2 cells are incubated with nicotine. These results indicate that mainly the mAchRs are involved in the inhibition of the NF-κB pathway in Caco-2 cells.

![Figure 1. NF-κB reporter assay. Caco-2 cells stably transfected with an NF-κB luciferase reporter construct were incubated with 25ng/ml IL-1β and 10, 100 or 1000 nM Ach, nicotine or muscarine for 16 hours. Thereafter, luciferase activity was measured as an indication of NF-κB activity.](image-url)
Acetylcholine inhibit the production of IL-8 via mAchR activation

Since Ach and muscarine are able to inhibit the NF-κB activity in Caco-2 cells, we wanted to investigate whether cholinergic neurotransmitters inhibit NF-κB-mediated IL-8 production by Caco-2 cells. Therefore, we used Caco-2 cells that were incubated for 20 minutes with increased concentrations of Ach, nicotine or muscarine and after 3 hours we measured the IL-8 production in the supernatant. Cells stimulated with Ach were also followed by incubation with 50nM neostigmine for three hours. Neostigmine inhibits the degradation of acetylcholinesterase, so that the Ach activity is prolonged. Besides neurones, also Caco-2 cells produce choline acetyltransferases\textsuperscript{37} and acetylcholinesterase\textsuperscript{38}, indicating that Caco-2 cells produce and break-down Ach themselves.

Caco-2 cells that were incubated with Ach or muscarine significantly produce less IL-8 in a concentration dependent manner compared to non-treated cells, whereas increased concentrations of nicotine does not change the IL-8 secretion (see figure 2). Also 1000nM Ach treated Caco-2 cells followed by a 3-hour incubation of 50nM neostigmine produce less IL-8 then non-treated cells (p<0.001).
Figure 2. Caco-2 cells were incubated for 20 minutes with 0, 100, 1000 or 10,000 nM Ach (a. and b.), nicotine (c.) or muscarine (d.). Three hours after this incubation, supernatant was collected and measured for IL-8 production. Figure b. shows the effect of 20 minutes incubation of Ach followed by three hours incubation of 50 nM neostigmine.
The production of IL-8 by Caco-2 cells is increased under influence of several cytokines such as TNF-α and IL-1β. To test whether also Ach, nicotine and muscarine inhibit cytokine-induced IL-8 production, Caco-2 cells were incubated with increased concentrations of Ach, nicotine or muscarine for 20 minutes followed by a 3 hour incubation of 1 or 100ng/ml TNF-α or 10 or 25ng/ml IL-1β. Thereafter the IL-8 production was measured in the supernatant. Mainly Ach and muscarine and not nicotine are able to inhibit significantly IL-8 production induced by low doses of TNF-α (1ng/ml), indicating that Ach acts via the mAchR on Caco-2 cells (see figure 3). This effect is exacerbated when Caco-2 cells are also incubated with neostigmine after Ach stimulation, probably because of the slowed break-down of intrinsic produced Ach. In case of high doses of TNF-α (100ng/ml), muscarine cannot inhibit the IL-8 production.

Ach, nicotine and muscarine significantly inhibit IL-8 production induced by IL-1β, although it seems that Ach prefers to act via nAchR when IL-1β concentrations are low (10ng/ml), since also 100 and 1000nM nicotine significantly decreases IL-8 production (p<0.01), whereas during high concentrations of IL-1β (25ng/ml) Ach acts via the mAchRs since then muscarine significantly decreases IL-8 production in a concentration-dependent manner (see figure 4). Probably, this inhibition of IL-1β-induced IL-8 production is dependent on a short action of Ach, because addition of neostigmine is less effective in inhibiting IL-8 production.
Figure 3. Caco-2 cells were incubated for 20 minutes with 0, 100, 1000 or 10,000 nM Ach (a., b., e. and f.), nicotine (c. and g.) or muscarine (d. and h.) followed by 1ng/ml (a., b., c. and d.) or 100ng/ml TNF-α incubation (e., f., g. and h.). Three hours after this incubation, supernatant was collected and measured for IL-8 production. The figures b. and f. show the effect of 20 minutes incubation of Ach followed by three hours incubation of 50 nM neostigmine together with 1ng/ml or 100ng/ml TNF-α respectively.
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Figure 4. Caco-2 cells were incubated for 20 minutes with 0, 100, 1000 or 10,000 nM Ach (a, b, e, and f), nicotine (c and g) or muscarine (d and h) followed by 10ng/ml (a, b, c, and d) or 25ng/ml IL-1β incubation (e, f, g, and h). Three hours after this incubation, supernatant was collected and measured for IL-8 production. The figures b and f show the effect of 20 minutes incubation of Ach followed by three hours incubation of 50 nM neostigmine together with 10ng/ml or 25ng/ml IL-1β respectively.
Acetylcholine reduces the permeability of Caco-2 monolayers

It has been described that several cytokines increase the intestinal epithelial permeability via activation of the NF-κB pathway. To investigate whether Ach influences the intestinal epithelial permeability, Caco-2 cells cultured on transwells were incubated with different concentrations of Ach with or without 50nM neostigmine followed by a cytokine mix which contains IL-1β, TNF-α and IFN-γ for 72 hour. After 72 hours the TEER was measured. Moreover, 4kDa FITC dextran particles were added at the mucosal site of the Caco-2 monolayer and the flux though the monolayer was measured after 30 and 60 minutes.

The TEER is decreased under influence of the cytokine mix correlating with an increase of 4kDa FITC dextran flux (see figure 5). Ach in the presence or absence of neostigmine did not change the TEER. However, Ach increases the dextran flux in a concentration-dependent manner, but in the presence of the cytokine mix, Ach decreases the dextran flux decreased after 60 minutes. Neostigmine has only an effect during the first 30 minutes of dextran flux.
Finally, we investigated whether Ach influences the expression of the TJ proteins occludin and ZO-1. After a 3 hour incubation of IL-1β and TNF-α, there is no translocation of occludin from the membrane to the cytosol under influence of different concentrations of Ach or muscarine (see figure 6). After 72h, the cytokine mix (IL-1β, TNF-α and IFN-γ) decreases the expression of ZO-1 in Caco-2 cells compared to the untreated cells (see figure 7). The expression of ZO-1 is not influenced by 100 or 1000nM Ach. However, when Caco-2 cells are treated with the cytokine mix, 100 and 1000nM Ach restore the ZO-1 expression.

**Figure 5.** TEER and 4kDa dextran flux measurements. After 72 hour of incubation with or without cytokine mix and different concentrations of Ach in the presence or absence of 50nM neostigmine, the TEER was measured (a.) and 4kDa FITC dextran particles were added at the mucosal site of the Caco-2 monolayer and the flux through the monolayer was measured after 30 (b.) and 60 minutes (c.).
Figure 6. Expression of occludin and ZO-1. After 3 hours of incubation of IL-1β and TNF-α, there is no translocation of occludin from the membrane to the cytosol under influence of different concentrations of Ach or muscarine (a.). After 72h, the cytokine mix (IL-1β, TNF-α and IFN-γ) decreases the expression of ZO-1 in Caco-2 cells (e.) compared to the untreated cells (b.). The expression of ZO-1 is not influenced by 100 or 1000nM Ach (d. and f.). However, when Caco-2 cells are treated with the cytokine mix, 100 and 1000nM Ach restore the ZO-1 expression (e. and g.).
Electrical vagal nerve stimulation enhances transepithelial transport under healthy conditions

Our *in vitro* data show that under inflammatory conditions Ach protects against epithelial barrier dysfunction. To investigate whether Ach also changes epithelial permeability under healthy conditions, the vagus nerve of mice was electrical stimulated after ligation of the distal part of the ileum. FITC-labelled *E. faecium* were injected in the ligated part of the ileum and after 30 minutes the mice were sacrificed so that the ileum could be analysed for translocation of *E. faecium* to the lamina propria and could be stained for lysosomal LAMP-2 to see whether there is co-localisation.

It seems that under healthy conditions stimulation of the vagus nerve increases paracellular transport of *E. faecium* compared to mice without VNS, since more FITC-labelled *E. faecium* was present in the lamina propria of VNS-treated mice (see figure 7). The uptake of *E. faecium* is not via lysosomes, because there is no co-localisation of *E. faecium* with LAMP-2.

*Figure 7.* The vagus nerve of mice was electrical stimulated after ligation of the distal part of the ileum where FITC-labelled *E. faecium* (green) was injected (b). Moreover, ileum was stained for lysosomal LAMP-2 (red). Mice without vagal nerve stimulation (VNS) were used as a control (a).
The aim of this study was to investigate the effect of Ach on the intestinal epithelial barrier integrity. We demonstrated that Ach reduces TNF-α or IL-1β induced IL-8 production of Caco-2 cells. Ach acts mainly via the mAchR, since only muscarine inhibits the IL-8 production and not nicotine, although nicotine also inhibits IL-8 production when it is induced by low concentrations of IL-1β (see figure 8). Nevertheless, only Ach and muscarine and not nicotine reduce IL-1β induced NF-κB activity of Caco-2 cells. Furthermore, we demonstrated that Ach reduces 4kDa FITC Dextran flux though Caco-2 monolayers and that Ach restores ZO-1 expression of Caco-2 cells after cytokine exposure. Our data implicate that Ach enforces the cellular processes leading to increased permeability and dendritic cell-mediated antigen uptake and drainage to the lymph nodes during homeostatic conditions, whereas under inflammatory conditions, Ach protects against cytokine-induced enhanced permeability to prevent excess exposure of the intestinal mucosa to luminal antigens and microbes.

It has been shown before that under inflammatory conditions activation of the vagus nerve improves the intestinal barrier integrity. In a rat model of haemorrhagic shock, a high fat diet which activates the vagus nerve via cholecystokinin, significantly reduces bacterial translocation, HRP permeability and rearrangement of ZO-1 39. Besides the improvement of the epithelial barrier integrity, vagus nerve activation reduces local and systemic inflammation 39,40. Moreover intracerebroventricular injection of ghrelin in a rat sepsis and ischemia-reperfusion
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model results in a reduction of intestinal epithelial permeability and bacterial translocation\(^{41,42}\). Since vagotomy inhibits the effects of ghrelin, this indicates that ghrelin improves mucosal barrier integrity through the vagus nerve.

Cameron and Perdue showed that transcellular transport is increased via the M3 muscarinic receptor under steady state conditions\(^{27}\). Nevertheless, mice that are deficient for the M3 muscarinic receptor are more susceptible for DSS-colitis\(^{43}\). Although it has been described that the M3 muscarinic receptor is involved in epithelial ion transport\(^{44-46}\), these M3 muscarinic receptor-deficient mice developed compensatory pathways. However, the colitis in these mice was characterised by the presence of inflammation in the ileum, whereas colitis normally is restricted to the colon and caecum. This indicates that the M3 muscarinic receptor has also anti-inflammatory capacities. Our data also demonstrate that muscarine is able to decrease NF-κB activity and IL-8 production by Caco-2 cells.

McGilligan et al. demonstrated that nicotine decreases epithelial gut permeability in Caco-2 cells\(^{47}\). Moreover, not only M3 muscarinic-deficient mice, but also α5 nAchR subunit-deficient mice are more susceptible for the development of colitis\(^{48}\). We could only find decreased NF-κB activity and IL-8 production of Caco-2 cells by Ach and muscarine, indicating that this effect is mediated via mAchRs. Probably, there is an effect of nicotine on the epithelial barrier integrity in Caco-2 cells, but not on the NF-κB activation and IL-8 production. That α5 nAchR subunit-deficient mice are more susceptible for the development of colitis may also be caused by an indirect effect on epithelial cells via interactions with enteric neurons and glial cells, which also influences epithelial barrier integrity.

Most likely, the vagus nerve does not interact directly with intestinal epithelial cells, but through enteric neurons and EGCs. EGCs produce S-nitroglutathione, which plays an important role in the homeostasis of the epithelial barrier. S-nitrosoglutathione is able to restore mucosal barrier function in colonic biopsies from CD patients\(^{49}\). Ablation of jenunal and ileal glial cells results in
inflammation of the intestine similar to IBD and reduces the intestinal barrier integrity by altering expression of perijunctional F-actin and association of ZO-1 and occludin with the actin-cytoskeleton. Moreover, EGCs were able to strongly inhibit intestinal epithelial cell proliferation in part by their release of transforming growth factor-β1 (TGF-β1). Interactions between the vagus nerve and EGCs seem to be important to maintain the epithelial barrier integrity under healthy conditions. However, also EGCs respond to inflammation by the production of IL-1β and TNF-α resulting in an increased epithelial permeability and activated immune cells. Probably, EGCs increase the epithelial permeability so that luminal antigens can enter the lamina propria where immune cells will take up and process these antigens to induce an inflammatory reaction. The release of Ach by enteric neurons or even epithelial cells themselves may inhibit this inflammation.

Together, our data on the potential of vagal motor activity to affect barrier function may be interpreted as a dual immune-supportive effect; enhancing immune surveillance and supporting tolerance under healthy immune homeostasis, while acting protective on inflammatory cytokine exposed epithelia. Firmly establish this hypothesis more data are required that pin-point the in vivo role of vagal nerve activity in support of gut immune homeostasis.
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