Ph.D. THESIS

INVESTIGATION OF THE PATHOMECHANISM OF DIARRHOEA-RELATED DISEASES

ÉVA PALLAGI

First Department of Medicine,
University of Szeged
Szeged, Hungary

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LIST OF FULL PAPERS NOT RELATED TO THE SUBJECT OF THE THESIS

I. K. Farkas, P.L. Lakatos, M. Szűcs, **É. Pallagi-Kunstár**, A. Bálint, F. Nagy, Z. Szepes, N. Vass, L. S Kiss, T. Wittmann, T. Molnár. Frequency and prognostic role of mucosal healing in patients with Crohn's disease and ulcerative colitis after one-year of biological therapy. *World J Gastroenterol* 2014 Mar 21;20(11):2995-3001

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LIST OF ABBREVIATIONS

$(\Delta\psi)_m$: mitochondrial transmembrane potential

$[Ca^{2+}]_i$: intracellular Ca^{2+}-concentration

ATI: antibody to infliximab

ATP_i: intracellular ATP-concentration

BA: bile acid

BAM: bile acid malabsorption

BAPTA-AM: 1,2-bis(o-aminophenoxy)ethane-N,N,N9,N9-tetraacetic acid

BCECF-AM: 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein-acetoxymethylester

CBE: Cl^-/HCO_3^--exchanger

CCCP: mitochondrial toxin carbonyl cyanide m-chlorophenyl hydrazone

CD: Crohn's disease

CDAI: Crohn's Disease Activity Index

CDC: non-conjugated bile acid chenodeoxycholic acid

D: ileum-resected/cholecystectomised patients suffering from diarrhoea

DOG/IAA: glycolysis inhitbitor deoxyglucose and idoacetamide

DRA: Cl^-/HCO_3^--exchanger downregulated in adenoma

FURA-2-AM: 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)-ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acetoxymethyl ester

GCDC: glycine-conjugated bile acid glycochenodeoxycholic acid

IBD: inflammatory bowel disease

IFX: infliximab

IP_3R: inositol triphosphate receptor

IQR: interquartile range

NHE: Na^+/H^+-exchanger

NON-D: ileum-resected/cholecystectomised patients without diarrhoea

pH_i: intracellular pH

RR: ruthenium red

RyR: ryanodin receptor

SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase

TEM: transmission electron microscopy

Tg: thapsigargin

TMRM: tetramethylrhodamine methyl ester

TNF-α: tumor necrosis factor alpha

UC: ulcerative colitis

I. INTRODUCTION

1. *The role of human colonic epithelial ion transporters in bile-acid induced diarrhoea*

The colon plays a fundamental role in the maintenance of the water balance of the body by absorbing 1.5 to 1.9 liters of electrolyte-rich fluid daily. The adequate activity of ion transporters of polarised epithelial cells is essential to keep the precise balance between absorption and secretion. The functionally coupled Na^+/H^+ exchanger 3 (NHE3) and Cl^-/HCO_3^--exchanger (CBE) downregulated in adenoma (DRA) are most probably responsible for the majority of electroneutral NaCl absorption in the colon. The presence of NHE2 and NHE3 on the apical membrane of colonic epithelial cells has been confirmed, however, only the prominent role of NHE3 in colonic Na^+ absorption has been demonstrated. The occurrence of diarrhoea in NHE3 knockout mice further supports the idea that this is the dominant NHE isoform responsible for Na^+ uptake in the intestine. Together with NHE3, the Cl^-/HCO_3^- exchanger DRA maintains the absorption of NaCl in the colon. Mutation of this transporter results in congenital chloride-losing diarrhoea, moreover, similar conditions develop in DRA-deficient mice. Disturbances in colonic epithelial Na^+ and/or Cl^- transport have shown previously to be involved in the development of diarrhoea in ulcerative colitis (UC) and secretory diarrhoea.

Bile acids (BAs) are natural detergents that participate in the solubilisation and absorption of dietary lipids. When their enterohepatic circulation impairs, bile acid malabsorption (BAM) occurs, thus BAs are allowed to enter the colon in higher concentration and can induce diarrhoea through unidentified mechanisms. BAM and diarrhoea are well-known clinical complications after ileal resection or after cholecystectomy. The prevalence of BAM is 97% in Crohn's patients with resection, 58% of patients with gastric surgery and/or cholecystectomy. Since the diagnosis is not available everywhere, the disease is under-recognised, therefore managing bile-induced diarrhoea is a great challenge for gastroenterologists. The therapy with the BA-sequestrant cholestyramine does not solve the problem in every case; on the other hand, a life-long medication of the patients is not supportable. Disturbed colonic absorptive and/or secretory functions, including Na^+ and Cl^- transport, must play a critical role in BAM-associated diarrhoea. BAs *in vivo* exert prosecretory and antiabsorptive actions on the human colon. Moreover, *in vitro* electrolyte secretion in cultured colonic epithelial cells is also stimulated by BAs. The mechanism, by which BAs induce diarrhoea, is not fully elucidated yet, therefore, to understand to pathogenesis of bile-induced diarrhoea and the development of new therapeutical approaches are extremely necessary.

2. Biological therapy with the anti-tumor necrosis factor-α (TNF-α) infliximab (IFX) in the management of inflammatory bowel diseases (IBD)

Crohn's disease (CD) and ulcerative colitis (UC) are the two types of inflammatory bowel diseases (IBDs). Genetic, environmental and immunological factors are thought to be involved in the development of the disease. IBD is characterized by alternating periods of relapse and remission. In

case of active disease, signs and symptoms beside diarrhoea may include abdominal pain and cramping, ulcers in the mouth, perianal fistulas, weight loss and fever.

The complexity of IBD makes a lot of difficulties in the treatment and an established standard therapy is lacking. In some of the cases, surgery is unavoidable. Beside 5-aminosalicylic acid compounds, corticosteroids and immunosuppressive drugs, biological therapies are most commonly used therapeutic methods. The proinflammatory cytokine TNF-α is the key target for the management of IBD. Infliximab (IFX), a chimeric monoclonal anti-TNF-α-antibody, has been approved for the induction and maintenance of remission in both CD and UC. Although IFX resulted in marked clinical improvement and macroscopic healing of the inflamed mucosa, loss of response, presence of antibodies against IFX, low drug serum concentrations, hypersensitivity and allergic reactions are proved to be predisposing factors for therapeutic failure. Approximately 40% of patients will subsequently lose response, thus requiring dose intensification or drug change. Dose intensification may be a solution in case of low anti-TNF-α drug trough levels, while switching to another drug could be useful if antibodies are developed against the biological agents. Immunogenicity (the formation of antibodies to the biological agents) is the major cause of loss of response and adverse reactions. Scheduled maintenance therapy, concomitant immunomodulators therapy, and pretreatment with high-dose corticosteroids may help to reduce immunogenicity. Many observational studies have linked low serum drug levels to a higher risk of the development of anti-drug-antibodies, and/or loss of response to biologics in IBD. Measurement of anti-drug-antibodies and serum drug levels and appropriate adjustment of drug regimen, has been utilized in practice to optimize clinical outcomes. Although the role of TNF-α measurement, together with antibody and drug serum concentration, has not previously been investigated in everyday practice, there are more and more studies emphasizing the importance of pharmacokinetic monitoring of IFX and anti-IFX-antibody (ATI) in order to prevent side-effects and to predict the clinical response to IFX and endoscopic improvement. In this regard, therapeutic drug monitoring may help to optimise the treatment of IBD-patients.

Although patients with IBD should not be routinely considered to have altered immunocompetence per se, there is currently no method of evaluating the effects of immunosuppression on the immune system. IBD-patients, receiving biological therapy and/or immunmodulators are exposed to an increased hazard for infectious diseases, with an incremental elevation in the relative risk of opportunistic infection: three fold increased risk if any one immunomodulator was used, increasing substantially if two or more drugs were used concomitantly. Some of the infections can be prevented with immunisation. Since influenza is one of the most common vaccine-preventable illnesses in adults, influenza vaccination is recommended for all IBD-patients on biological therapy and/or immunmodulators. The types of immunosuppressive and biological therapies seem to affect the immune response to vaccinations, but it remains unclear, whether vaccination has an impact on the cytokine profile of IBD-patients, by which it may influence the process of the disease.

II. AIMS

1. To investigate the influence of bile acids on the ion-transporter activities of human colonic epithelial cells and to characterise the cellular pathomechanism of bile-induced diarrhoea.

2.1. To assess tumor necrosis factor-a (TNF-a), infliximab (IFX) concentrations, and antibodies against IFX in patients with inflammatory bowel disease (IBD) who develop loss of response, side effects, or allergic reaction during anti TNF-α therapy.

2.2. To evaluate the cell-mediated immune response to split and whole virion influenza vaccines in patients with IBD treated with anti-TNF-α and/or immunosuppressive therapy.

III. MATERIALS AND METHODS

1. The role of human colonic epithelial ion transporters in bile-acid induced diarrhoea

1.1. Patients enrolled in the study

Beside control patients, ileum-resected/cholecystectomised patients with or without diarrhoea were involved in the study. In none of the patients were any sign of IBD detected. An informed consent was obtained prior to endoscopy. Protocols of the study were approved by the regional ethical committees. Six colonic biopsies were obtained from each patient undergoing colonoscopy.

1.2. Isolation of colonic crypts

Colonic crypts were isolated from 6 biopsy specimens of control and UC patients. After 2 times 30 minutes enzymatic digestion with 0.38 mg/ml collagenase the crypts were aspirated into a micropipette and transferred to a Petri dish.

1.3. Measurement of pH_i, $[Ca^{2+}]_i$, $(ATP)_i$ and $(\Delta\Psi)_m$

Isolated human colonic crypts were incubated in standard HEPES solution and loaded with BCECF-AM (1.5µmol/L), Fura2-AM (2.5µmol/L), MgGreen-AM (5µmol/L), or TMRM (100nmol/L) respectively for 30 min at 37°C. The crypts were continuously perfused with solutions and were excited with light at adequate wavelengths during the microfluorometric measurements. Fluorescence emission ratio was detected. For $(\Delta\Psi)_m$ measurements glass bottom petri dishes were perfused continuously with solutions containing 100 nmol/L TMRM at 37^{o}C at a rate of 2-2.5 ml/min.

1.4. Determination of NHE activities

The crypts were acid loaded by exposure to a 5-min-pulse of 20 mM NH_4Cl in HEPES solution followed by a 10-min-exposure of Na^+-free HEPES solution. NHE activity was switched on

by re-addition of extracellular sodium and the activity of NHE was determined by measuring the initial rate of pH_i recovery over the first 60 sec. The activities of the different NHE isoforms were extracted by using the isoform selective NHE inhibitor HOE-642. 1 µM HOE642 inhibits NHE1 whereas 50 µM HOE642 inhibits both NHE1 and 2 but not NHE3.

1.5. Measurement of CBE activity

The Cl^-/HCO_3^- exchange activity of the cells was determined using the Cl^- withdrawal technique. Briefly, removing Cl^- from the standard HCO_3^-/CO_2 buffered solution causes alkalization due to the reverse activity of the Cl^-/HCO_3^- exchanger. The activity of the exchanger was determined by measuring the initial rate of alkalization over the first 30 sec.

1.6. Determination of buffering capacity and base efflux

The total buffering capacity (β_{total}) was estimated according to the NH_4^+ pre-pulse technique. Colonic epithelial cells were exposed to various concentrations of NH_4Cl in a Na^+- and HCO_3^--free solution. β_i (which refers to the ability of intrinsic cellular components to buffer changes of pH_i) was estimated by the Henderson–Hasselbach equation. β_{total} was calculated from: $\beta_{total} = \beta_i + \beta_{HCO3-} = \beta_i + 2.3x[HCO_3^-]_i$, where β_{HCO3-} is the buffering capacity of the HCO_3^-/CO_2 system. The measured rates of pH_i change (dpH/dt) were converted to transmembrane base flux $J(B^-)$ using the equation: $J(B^-)=dpH/dtx\ \beta_{total}$. The β_{total} value at the start point pH_i was used for the calculation of $J(B^-)$. We denote base influx as $J(B^-)$ and base efflux (secretion) as $-J(B^-)$.

1.7. Electron microscopy

Biopsy samples were fixed in 2% glutarldehyde (in PBS). Samples were cut into small pieces (1X1 mm), infiltrated with 2% gelatin (PBS) and embedded to Embed 812 (EMS, USA). Thin (70nm) sections were cut for transmission electron microscopy (TEM) examination.

1.8. Statistical analysis

Values are means ± SE. Statistical analyses were performed using analysis of variance (ANOVA) with the post-hoc test Dunnett or Bonferroni. $P \leq 0.05$ was accepted as significant.

2. Biological therapy with anti-TNF-α IFX in the management of IBD

2.1. Study population I.

In the first part of this prospective observational clinical study, 67 patients with CD and UC treated with IFX were enrolled. Blood samples of 36 patients with response loss, side effects, or hypersensitivity to IFX therapy (Group I) and 31 patients in complete clinical remission (Group Ⅱ) selected as a control group were collected to measure trough serum TNF-α level, IFX, and anti-IFX

antibody (ATI) concentration. The study was approved by the Regional and Institutional Human Medical Biological Research Ethics Committee of the University of Szeged. We examined the correlation between loss of response, side effects, or hypersensitivity and serum TNF-α, IFX trough levels, and ATI concentrations.

2.2. Study population II.

The second section was a multicentre, prospective cohort study at 4 Hungarian IBD centres. Inclusion criteria included an age ≥ 18 years, diagnosis of IBD stable for more than 3 months, no signs of activity (biological and clinical) and not requiring any treatment modification for the disease at inclusion. Influenza vaccination was offered to every patient. Patients were randomised to two groups on the basis of the acceptance of the vaccination. Patients refusing the vaccination served as controls. Patients and controls were followed up for 4 months. Patients who received vaccination were divided into two further groups: patients treated with aminosalicylates without immunosuppressive therapy and patients treated with immunomodulator and/or biological therapy for at least three month before the vaccination. Controls had received maintenance therapy with immunomodulator and/or biological therapy for at least three month before the vaccination.

The type of vaccine (whole virion or split virion vaccine) was randomly selected. Blood samples were taken before and after the vaccination. Ethical approvals for the study had been obtained from the Scientific and Research Ethics Committee of Hungary. Written informed consent was obtained from each subject.

2.3. Vaccines

Two non-live vaccines directed against the seasonal influenza virus A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), B/Wisconsin/1/2010–like B/Hubei-Wujiagang/158/2009 were used. Inactivated, split virion vaccine (IDFlu9) and inactivated, whole virion vaccine (Fluval AB) were administrated depending on a random selection.

2.4. Measurement of serum IFX trough levels, and ATI concentrations, TNF-α, IFN-γ and IL-2 levels

Enzyme-linked immunosorbent assay (ELISA) was applied to determine the serum levels of TNF-α, IFX trough levels, and ATI concentration. Blood samples, from IBD-patients, participating in the first part of our clinical study, were obtained prior to application of IFX infusion.

In the second part of our clinical study, serum was collected at pre-vaccination and 5 to 6 weeks after vaccination. We assessed cell-mediated immune response using an INF-γ, IL-2, and TNF-α ELISA after vaccination and also compared it between patients treated with and without immunosuppressants.

2.5. Statistical analysis

In order to examine the correlation between loss of response, the development of side effects or hypersensitivity, and serum TNF-α, IFX trough levels, and ATI concentrations, medians with an interquartile range (IQR), Pearson χ 2 statistic, Mann-Whitney *U* and Fisher's exact tests were used respectively. A *P* value less than 0.05 was considered to be significant.

To the evaluation of cell-mediated immune response to the split and whole virion influenza vaccine in patients with IBD treated with anti-TNF-α and/or immunosuppressive therapy, data were analysed using SPSS version 21 software (SPSS, Chicago, IL). $p<0.05$ was considered significant. Pearson's chi-square, Fisher's exact test, multivariate analysis of variance (MANOVA) models were used. p-values were corrected with the Holm-Sidak method.

IV. RESULTS

1. The role of human colonic epithelial ion transporters in bile-acid induced diarrhoea

1.1. Chronic exposure of the colon to BAs impair the activities of NHEs and CBE of human colonic epithelial cells

The functions of all examined NHE isoforms and CBE were significantly reduced in colonic crypts isolated from ileum-resected or cholecystectomised patients suffering from diarrhoea, compared to control patients. In colonic crypt cells, isolated from patients, who did not develop diarrhoea after the surgical intervention, the activities of the examined acid/base transporters were not changed significantly, compared to the control group, suggesting the significant role of ion transporters in bile-induced diarrhoea.

1.2. Effects of BAs on the pH_i of isolated human colonic epithelial cells

The administration of the non-conjugated chenodeoxycholate (CDC) and the conjugated glycochenodeoxycholate (GCDC) dose-dependently reduced the pH_i of perfused colonic epithelial cells, isolated from control patients. The influx of BAs was markedly greater when the non-conjugated CDC. This could be due to the lipophilic property of non-conjugated BAs which allows them to permeate through the membrane.

1.3. Short-term influence of BAs on the activities of acid/base transporters of isolated human colonic epithelial cells

Colonic crypts isolated from control patients. In order to investigate the effects of BAs on the activities of NHEs, we analysed the pH_i recovery from an acid load induced by the removal of NH_4Cl. 10 min treatment with 0.1 mM CDC or GCDC had no effect on the functions of NHEs. 0.3 mM CDC reduced the activities of all of the examined NHE isoforms. In addition, treating the crypts with

0.3mM CDC resulted in a strong inhibition in the activity of CBE. Neither low concentration (0.1 mM) of CDC nor 0.1 mM or 0.3 mM GCDC influenced the function of CBE.

1.4. Effects of BAs on the intracellular morphology of isolated human colonic epithelial cells

TEM showed that low concentration of CDC (0.1 mM or 0.3 mM) or 1 mM GCDC for 1-10 min had no effect on the structure of intracellular organelles. High concentration (1 mM) of CDC strongly damaged all of the mitochondria. Other intracellular organelles remained unaltered.

1.5. Effects of CDC on ATP_i and $(\Delta\psi)_m$ of isolated human colonic epithelial cells

0.3mM CDC significantly, but reversibly depleted ATP_i of isolated human colonic epithelial cells. Both the mitochondrial and the glycolytic ATP production were break down.

0.1mM or 0.3mM CDC induced the reversible loss of $(\Delta\psi)_m$.

1.6. CDC dose dependently increases $[Ca^{2+}]_i$ via endoplasmic reticulum (ER) Ca^{2+} release and extracellular Ca^{2+} influx

CDC caused a dose-dependent, sustained, plateau-like pathophysiological increase in $[Ca^{2+}]_i$. This effect was significantly inhibited by caffeine, the antagonist of inositol triphosphate receptor (IP_3R). Gadolinium (Gd^{3+}, 1μM) was applied to block plasma membrane Ca^{2+} entry channels. Gd^{3+} alone was not able to decrease the elevation of $[Ca^{2+}]_i$ induced by 0.3mM CDC.

In Ca^{2+}-free solution, the SERCA inhibitor Tg induced Ca^{2+} store depletion with consequent $[Ca^{2+}]_i$ elevation. This elevation was markedly decreased when Tg was applied after 0.3 mM CDC. When administered during 0.3mM CDC, Tg further induced a slight increase in $[Ca^{2+}]_i$. These observations suggest that beside the extracellular Ca^{2+} influx, CDC deplete ER Ca^{2+}-stores via IP_3R mediated processes.

1.7. CDC induces ATP-dependent decrease in the NHE-activities and Ca^{2+}-dependent inhibition of CBE activity in isolated human colonic epithelial cells

The potential connection was examined between the intracellular effects of 0.3mM CDC (ATP_i depletion and $[Ca^{2+}]_i$ elevation) and the decreased function of acid/base transporters following treatment with the BA. The glycolysis inhibitors DOG+IAA had the same inhibitory effect on NHEs as 0.3 mM CDC, which suggests that CDC inhibits NHE via ATP_i-depletion. The intracellular Ca^{2+}-chelator BAPTA-AM completely abolished the inhibitory effect of 0.3mM CDC on the activity of CBE, which reveals the strong Ca^{2+}-dependence of the transporter.

2. Biological therapy with the anti-TNF-α IFX in the management of IBD

2.1. Assessment of TNF-α, IFX concentrations, and antibodies against IFX in IBD-patients who develop loss of response, side effects, or allergic reaction during anti TNF-a therapy

The median CDAI in groups I and II were 138 (IQR 68-186) and 50 (IQR 34-70), respectively; the partial Mayo score in the two groups were 5 (IQR 3-6) and 1 (IQR 0-1), respectively. The median serum TNF-α levels were 10.5 (IQR 3.2-18-9) and 6.3 (IQR 1.5-15.7) pg/mL in groups I and II, respectively. The median IFX trough level was 3.1 (IQR 2.6-5.04) and 3.5 (IQR 2.6-4.7) μg/mL in the two groups, respectively. Fourteen patients were found to have ATI positivity with a median of 933 μg/mL (IQR 328-3306). ROC analysis revealed that the cut off value of serum IFX for detecting ATI was 3.01 μg/mL. The serum TNF-α level was significantly higher in the presence of ATI (24.23 pg/mL *vs* 6.28 pg/mL, $P = 0.005$). ATI positivity correlated significantly with low trough levels of IFX (2.66 μg/mL *vs* 3.86 μg/mL, $P = 0.015$). However, no difference was detected in serum IFX and antibody levels between the two groups (2.67 μg/mL *vs* 2.66 μg/mL, $P = 0.821$). Serum IFX and ATI levels in patients with ATI positivity are summarized in Table 3.

Two of the IBD patients with antibodies against anti TNF-α developed side effects, 5 patients lost response, and an allergic reaction occurred in 3 patients. 37 patients were previously treated with biologicals, with development of ATI being more frequent those patients ($P = 0.048$). Dose intensification was required in 9 patients. No association was found between dose intensification and the development of ATI. Concomitant immunosuppression had no impact on IFX trough levels or on the development of ATI formation. Increased erythrocyte sedimentation rate (ESR) and C-reactive protein correlated significantly with lower serum IFX level ($P = 0.04$ and $P = 0.002$). The serum TNF-α level was higher in patients not treated concomitantly with steroids ($P = 0.038$).

2.2. The impact of influenza-vaccination on the cellular immune response of IBD-patients treated with anti-TNF-α and/or immunosuppressive therapy.

209 IBD patients (127 with CD, 82 with UC) were eligible and enrolled in the study. 156 patients received influenza vaccination, whereas 53 patients (control group) refused the vaccine. Whole virion vaccine was given to 57; split virion vaccine was given to 99 patients. Out of the 156 vaccinated patients, 98 had CD, 58 had UC. Median disease duration was 9 years for CD (IQR 5-13), and 9 years for UC (IQR 4-15.8). Of the control subjects, 29 had CD and 24 had UC. Median disease duration was 7 years for both CD (IQR 5-14) and UC (IQR 4.5-12).

Out of the 156 vaccinated patients, 115 received immunosuppressive therapy. The non immunosuppressive group of vaccinated subjects was composed of 41 patients. Out of the 53 control subjects, 32 received immunosuppressive therapy. Twenty-one patients were free of immunosuppressive therapy. 8.3% of the patients were regularly vaccinated against seasonal influenza virus. 39 patients (21.5%) had received the last vaccination within one year, 25 patients (13.8%)

within 3 years and 3 patients (1.7%) within 5 years. 63% of the patients had received the last vaccination more than 5 years earlier.

Leukocyte and lymphocyte levels did not differ significantly after vaccination. Neither TNF-α, nor INF-γ levels changed significantly after influenza vaccination; however, a significant decrease was observed in the level of IL-2 after vaccination with split vs. whole virion vaccine (p=0.004).

V. DISCUSSION

1. The role of human colonic epithelial ion transporters in bile-acid induced diarrhoea

In this study, we provide evidence, that BAs impair the activity of the acid/base transporters (NHE and CBE) via different mechanisms in human colonic epithelial cells. The impaired activities of NHE and CBE can decrease the fluid and electrolyte absorption in the colon and promote the development of diarrhoea.

The absorption of water and ions (especially Na^+ and Cl^-) is an important function of colonic epithelial cells. The electroneutral NaCl absorption is probably mediated via the coupled activity of NHEs and CBE. Impaired activities of these transporters were observed in diarrhoea-associated diseases.

BAs are known to induce diarrhoea, which is a common feature of BAM. BAM often develops after small bowel resection or post-cholecystectomy. The influence of BAs on the absorptive and secretory function of colonic epithelium is supposed to be critical in the development of bile-induced diarrhoea, however, its exact pathogenesis is not yet completely understood.

First, we clarified, that the decreased activities of acid/base transporters are involved in bile-induced diarrhoea. We isolated colonic crypts from ileum-resected or cholecystectomised patients, whose colon is probably continuously exposed to high concentration of BAs. Patients were divided into two groups depending on having (Diarrhoea - D) or not having diarrhoea (Non-Diarrhoea – NON-D) after the surgical intervention. In the D-group, the activities of all examined NHE isoforms and the function of CBE were markedly lower than in control patients. The functions of the examined acid/base transporters were unaltered in colonic epithelial cells isolated from biopsy samples of NON-D patients. These data suggest that the reduced absorptive function of the colon is probably due to the continuous presence of non-physiological concentration of BAs.

Next, we characterised the basic effects BAs on human colonic epithelial cells. Colonic crypts were isolated from control patients and were treated with the non-conjugated BA CDC and the conjugated GCDC. Due to the conjugation, the natures of the BAs are changing. Being a weak acid, CDC can traverse cell membrane by passive diffusion, whereas conjugated BAs require active transport mechanisms for cellular uptake. Three concentrations of the BAs were applied (0.1, 0.3 and 1 mM) in order to imitate physiological and non-physiological circumstances. BAs caused an

immediate, dose-dependent and reversible decrease of the pH_i, which acidosis was more prominent in case of the CDC, due to the typical characteristics of the non-conjugated BAs.

We also investigated the short-term effects of BAs on the functions of ion transporters of healthy colonic epithelial cells. CDC in a relative high concentration (0.3 mM) caused a significant inhibition of NHEs and CBE of human colonic epithelial cells, suggesting the possible toxic effects of high doses of non-conjugated BAs. The functions of all examined NHE isoforms were reduced. Although the secretion of Cl^- and Na^+ in response to BAs was not investigated in this study, it is already well established that BAs stimulate intestinal electrolyte and fluid secretion and the decreased absorption and elevated secretion of ions may account for the diarrhoea associated with BAM.

The question raised how BAs are able to explain this inhibitory effect on NHE and CBE. First, we investigated the effects of BAs on the morphology of intracellular organelles. 1 mM CDC caused a severe damage in all of the mitochondria. The mitochondria swelled up and the structure of the inner membranes was lost. Other intracellular organelles seemed to remain intact. Although 0.3 mM CDC inhibited the activities of acid/base transporters, but it did not induce alteration in the structure of intracellular organelles of human colonic epithelial cells.

We decided to investigate mitochondrial transmembrane potential $(\Delta\psi)_m$ as well, being the essential driving force for ATP synthesis. 0.1 mM and 0.3 mM CDC resulted in the reversible loss of $\Delta\psi_m$. Although 0.3 mM CDC is not enough to cause structural damage of the cell compartments, it is still able to deplete ATP_i and diminish $\Delta\psi_m$ by which perturbs the energy homeostasis of human colonic epithelial cells. These processes may play a role in the impaired function of ion absorption.

Next, we investigated another potential intracellular target of BAs, the Ca^{2+} signalisation. It was shown, that the CBE and NHE3 are inhibited by the pathological increase of intracellular Ca^{2+}. In our experiments, CDC dose-dependently induced a sustained non-physiological elevation of $[Ca^{2+}]_i$, with a plateau-characteristic. The source of Ca^{2+} could be the ER through IP_3R and/or RyR or the extracellular space. In order to identify the orignin of elevated Ca^{2+} during administration of 0.3 mM CDC, the IP_3R antagonist caffeine, the RyR-blocker RR and the plasma membrane Ca^{2+} channel inhibitor gadolinium (Gd^{3+}) were applied. In our experiments, caffeine reduced the $[Ca^{2+}]_i$ elevation, induced by 0.3 mM CDC. Neither RR, nor Gd^{3+} alone were able to prevent the toxic Ca^{2+} signal, suggesting that CDC mobilizes stored Ca^{2+} from the ER via IP_3R. Since the inhibition of IP_3R did not completely abolished the effect of CDC, a Gd^{3+} insensitive extracellular Ca^{2+} influx must be present as well. Inhibition of SERCA induced a further elevation of the $[Ca^{2+}]_i$ after/during CDC administration, suggesting that CDC does not completely empty the ER Ca^{2+} store. These observations lead us to the hypothesis that CDC mobilizes stored Ca^{2+} from the ER and promotes the influx of external Ca^{2+}.

Finally, we examined the conjunction between the inhibitory effect of 0.3 mM CDC on the activities of acid/base transporters and its intracellular actions on ATP_i or $[Ca^{2+}]_i$. We measured again the ion transporter activities of human colonic crypts, isolated from patients with healthy colon. Depletion of ATP_i resulted in a similar decrease of the activities of NHEs as it was perceptible

following administration of 0.3 mM CDC. In contrast, pretreatment of the colonic epithelial cells with the $Ca^{2+}{}_i$ chelator BAPTA-AM did not prevent the toxic effect of 0.3 mM CDC on the activities of NHEs. These results indicate that 0.3 mM CDC inhibits the functions of NHEs via depleting ATP_i. This is in agreement with previous observations that NHEs are secondary-active transporters and their function is ATP-dependent. ATP_i depletion did not have any influence on the Cl^- absorptive capacity of colonic epithelial cells. Preventing the 0.3 mM CDC-induced sustained elevation of $[Ca^{2+}]_i$ with BAPTA-AM, restored the decreased activity of CBE. This observation supports the hypothesis that CBE is inhibited by the non-physiological elevation of $[Ca^{2+}]_i$.

Our results might contribute to the development of new therapeutical approaches in the treatment of bile-induced diarrhoea.

2. Biological therapy with the anti-TNF-α IFX in the management of IBD

Biological therapy revolutionized the treatment of IBD, but a significant proportion of patients loses response to these agents or develops adverse effects during the treatment. Parallel treatment with steroids and/or immunomodulators may help to avoid unfavourable outcomes. Loss of response occurs mostly due to the phenomenon of immunogenicity,which leads to subtherapeutic drug concentrations. Immunogenicity induced by IFX can be determined by monitoring the serum concentrations of ATI, TNF-α and IFX. The formation of ATIs results in a decreased level of serum IFX, increased risk of infusion reactions and diminished clinical response. Maintenance vs. episodical IFX therapy or concomitant immunomodulators are beneficial therapeutical strategies reducing ATI development and the risk of infusion reactions. Interestingly, antibodies against IFX Fab fragment are detectable in patients never received IFX therapy, and may predict long-term clinical efficacy and safety of IFX. Measurement of these antibodies before IFX treatment might help to select the best type of therapeutic TNF-blocker in individual patients.

In our study both increased TNF-α and decreased IFX levels correlated with the presence of ATI, although neither ATI nor serum IFX influenced the outcome of the therapy. A meta-analysis also concluded that the presence of ATIs is associated with a significantly higher risk of loss of clinical response to IFX and lower serum IFX levels. Although these statements are logical, the results of the clinical practice are confusing. Steenholdt et al. found that improved clinical outcome was associated with a higher increase in IFX levels. Chaparro published that there is a close relationship between trough levels of anti-TNF-α and maintenance of response. Although higher serum IFX level proved to predict longer duration of response and clinical remission, a Japanese study showed that the median trough levels of IFX did not differ significantly in patients who maintained and who lost response to IFX. In our study, previous biological therapy had more significant effect on the outcome of IFX therapy than the concomitant use of thiopurines. According to the study of Afif et al., dose escalation was associated with a high clinical response in patients with subtherapeutical IFX levels and negative ATI, and better clinical outcome was achieved in ATI positive patients switching to another anti-TNF-

α-drug. On the basis of previous studies, concomitant corticosteroid therapy is suggested to decrease the effect of TNF-α blocker confirmed by our results regarding the higher TNF-α level in patients receiving steroids.

Because of these controversial data, the usefulness of monitoring the trough levels and ATI concentrations in the therapeutic decisions may be questionable. Our results do not confirm the clinical utility of trough level and antibody measurement in the differentiation of "problematic' patients with loss of response of adverse reactions vs. those who respond appropriately to the biological therapy. Anyway, therapeutic drug monitoring could be a key device in the optimised management of IBD patients, however, the methods should be correctly standardised and individualised, which provides cost-effectiveness as well. The same goal has the Trough level Adapted infliXImab Treatment (TAXIT) trial. Furthermore, Vande Casteele et al. revealed, that IFX-dosing based on therapeutic drug monitoring was associated with fewer flares during the course of treatment, in comparison with clinically based drug-dosing.

In conclusion, in our prospective observational study we found significant association between serum TNF-α level and the presence of ATI; and also between ATI positivity and low trough levels of IFX. However, antibody positivity and lower serum IFX levels did not correlate with loss of response, side-effects and hypersensitivity. Previous use of IFX correlated with the development of ATI. On the basis of the present work, we suggest that further prospective studies are needed to determine whether the simultaneous measurement of serum TNF-α level, serum anti TNF-α concentration and antibodies against anti TNF-α may help to optimize the therapy in the critical situations.

Patients on immunosuppression or biological therapy are supposed to be at increased risk of influenza, since immunotherapy is known to predominantly impair cellular immunity, leaving the humoral immune response more or less intact. Thus, annual influenza vaccination is recommended for all patients with IBD on immunomodulators. In the second part of our study, we examined whether influenza vaccination has an effect on the cell-mediated immune response by measuring the pre- and post-immunisation levels of INF-γ, IL-2 and TNF-α. Interestingly, only the level of IL-2 decreased significantly after vaccination. Holvast et al. found that the frequencies of CD4+ T cells producing TNF and IL-2 were lower in patients after vaccination compared with healthy control subjects. They also found that this diminished cell-mediated response may reflect the effects of concomitant use of immunosuppressive drugs. Our results suggest that IBD patients on immunosuppressive therapy are recommended to be immunised against influenza, but larger and more detailed studies are needed to examine the cell-mediated response and to determine the efficacy of influenza vaccination in immunocompromised IBD-patients.

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