Final Report

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Analysing the role of COX-2 and alpha2-adrenoceptors in NSAID-induced small intestinal injury and dysbiosis

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1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) belong to the most commonly used medications worldwide, but they can induce significant damage to the gastrointestinal (GI) tract, including the small intestine. This so-called enteropathy can occur in up to 70% of chronic NSAID users, and has a complex pathogenesis including inhibition of cyclooxygenase (COX)--mediated prostaglandin synthesis and changes in the composition of intestinal bacteria (dysbiosis) and bile acids [1-3]. In contrast to NSAID-induced gastropathy there are no proven ways of either preventing or treating enteropathy.

Over the past decades considerable efforts have been made to identify the pathophysiological effects of NSAIDs leading to small bowel damage, but there are still several questions and apparent contradictions in the literature. Does the chronic use of selective COX-2 inhibitors causes also enteropathy [4,5], or are these agents safer than the non-selective NSAIDs [6,7]? Do NSAIDs cause intestinal dysbiosis by damaging the intestine and causing inflammation [8], or does dysbiosis develop in the non-inflamed gut as well [9]? Do selective COX-2 inhibitors cause dysbiosis [10] or not [11]? Does COX-2 inhibition aggravate [12] or reduce [13] intestinal damage following an ischemic insult? Can we treat enteropathy with gastroprotective agents, like alpha2-adrenoceptor agonists [14]? The present project was designed to answer (at least partly) these questions.

2. Results

2.1. 1st subproject – Analysing the effect of NSAIDs on small intestinal mucosal integrity and microbial composition

In this subproject male Wistar rats were treated with different NSAIDs (either non-selective or selective for COX-1 or COX-2) for 4 weeks. Then, the small intestines were harvested and the severity of enteropathy, as well as the composition of small intestinal microbiota and bile acids were evaluated. Although it was originally not planned, we have also performed a subacute study, in which rats were treated with a single high dose of indomethacin, and intestinal samples were harvested 24, 48 and 72 h after the treatment. These samples were thereafter used as positive controls, and also allowed us to determine the time-course of changes induced by COX inhibition.

a) We have demonstrated that chronic treatment with the selective COX-2 inhibitor rofecoxib does not cause enteropathy and intestinal dysbiosis in the rat. Because these results contrast with those reported in celecoxib- and etoricoxib-treated animals [5,10,15], we concluded that **chronic inhibition of COX-2 *per se* does not likely induce mucosal damage or microbial alterations**, and such effects caused by celecoxib and etoricoxib may be attributed to other drug-specific properties, like topical irritancy or direct antibacterial effects.

These results were presented at both international (World Congress of Pharmacology 2018) and Hungarian conferences (60th Annual Meeting of the Hungarian Society of Gastroenterology 2018, Semmelweis University PhD Scientific Days 2018), and were finally published in Cells journal.

Lázár B et al. Lack of Small Intestinal Dysbiosis Following Long-Term Selective Inhibition of Cyclooxygenase-2 by Rofecoxib in the Rat. Cells. 2019 8(3):251. IF.: 4.366

b) In our subsequent studies, we found similar results with celecoxib. These support our previous conclusion that chronic inhibition of COX-2 does not likely induce mucosal damage or small intestinal dysbiosis in the rat. In addition, although celecoxib possesses direct antimicrobial properties *in vitro*, this probably does not have any significant effect on the composition of microbiota *in vivo*. Our results are in contrast to those of [5] and [10], but it is important to note that we used celecoxib at doses of 3 and 30 mg/kg (which both inhibited COX-2 activity selectively and by more than 90%), whereas mucosal damage [5] and dysbiosis [10] were reported at a higher dose (100 mg/kg). It is also possible that species differences account for the different outcomes, as the studies reported in [5] and [10] were conducted on mice.

Interestingly, despite having no major effect on the composition of microbiota, celecoxib induced profound changes in the intestinal bile acid metabolome, which was mainly characterized by the increased proportion of hyodeoxycholic acid (HDCA). Of note, we found similar results with etoricoxib. Hence, **although selective COX-2 inhibitors may spare the GI mucosa, they may still able to induce important GI (and systemic) effects via altering the bile acid metabolome**. For example, the increased luminal concentration of HDCA may induce glucose-lowering and obesity-preventing effects [16].

Some parts of these results were presented at Hungarian conferences (61th Annual Meeting of the Hungarian Society of Gastroenterology 2019, Students' Scientific Congress at Semmelweis University 2021), and are also planned to be presented at the 11th International Symposium on Cell/Tissue Injury and Cytoprotection/Organoprotection /ISCTICO/ (which was postponed from 2020. October to 2021. October due to the COVID pandemia). In addition, a manuscript is under preparation.

c) Ketorolac, a COX-1-preferential inhibitor, was used mainly as a positive control. However, because low doses of ketorolac (< 3 mg/kg) spare the gastric and intestinal mucosa in the rat [17], and ketorolac has no direct antibacterial effects *in vitro* [18], it also proved to be a useful pharmacological tool to analyse the pathogenesis of enteropathy. Our studies confirmed that low-dose ketorolac does not cause enteropathy, but we have demonstrated for the first time that it induced small intestinal dysbiosis, which resembled that caused by other NSAIDs. Namely, it decreased the abundances of bacterial families belonging to Firmicutes, and increased that of *Enterobacteriaceae*. These changes were accompanied by and correlated with significant bile acid alterations. GI transit was not influenced significantly by ketorolac. These results indicate that **NSAIDs can alter the composition of intestinal microbiota and bile acids without causing intestinal inflammation or delaying intestinal peristalsis**, and other, yet unidentied, factors may also contribute to NSAID-induced dysbiosis and bile dysmetabolism.

Hutka B et al. The Nonsteroidal Anti-Inflammatory Drug Ketorolac Alters the Small Intestinal Microbiota and Bile Acids Without Inducing Intestinal Damage or Delaying Peristalsis in the Rat. Front Pharmacol. 2021 12:664177. IF.: 5.81

d) Although the non-selective COX inhibitor indomethacin was also used mainly as a positive control, we have performed a subacute study with a single high dose of indomethacin, and harvested the intestinal samples at different time points in order to determine the time-course of dysbiosis and bile dysmetabolism. We found that indomethacin induced marked changes in ileal bile acid composition, which were mainly characterized by a shift towards a higher proportion of conjugated bile acids. Interestingly, indomethacin did not increase the overall hydrophobicity of ileal bile, which was proposed previously by other researchers as a potential mechanism for NSAID-induced intestinal damage [1, 19]. **We have also identified notable differences in the temporal changes of Gram negative bacteria** (Gammaproteobacteria increased first, but declined later and were partially replaced by *Bilophila*, *Bacteroides* and *Fusobacterium*), **which may explain some apparent contradictions between earlier studies**. Furthermore, our analysis revealed numerous significant associations between inflammatory markers, bile acids and bacteria, which may help to decipher the complex pathogenesis of NSAID-enteropathy.

Lázár B et al. A comprehensive time course and correlation analysis of indomethacin-induced inflammation, bile acid alterations and dysbiosis in the rat small intestine. Biochem Pharmacol. 2021 190:114590. IF.: 5.858

- Besides NSAIDs also opioids (the other major class of painkillers) can induce intestinal dysbiosis, and there is some evidence that this may contribute to the development of analgesic tolerance. I've discussed these points in a review paper written together with my colleagues Dr. Zsuzsanna Fürst and Dr. Mahmoud Al-Khrasani.

Fürst S et al. On the Role of Peripheral Sensory and Gut Mu Opioid Receptors: Peripheral Analgesia and Tolerance. Molecules. 2020 25(11):2473. IF.: 4.411

2.2. 2nd subproject – Analysing the effect of selective COX-2 inhibitors on ischemia/reperfusion-induced small intestinal damage

Although rapid upregulation of COX-2 in response to I/R has been demonstrated in numerous organs including the GI tract [13], it is still not clear whether this represents an adaptive response that protects the cells from I/R injury, or it is rather detrimantal by promoting the inflammatory reaction and tissue damage. In this subproject rats were treated chronically with either vehicle or with selective COX-2 inhibitors, and then they were subjected to transient (30 min) occlusion of the superior mesenteric artery followed by 2 h of reperfusion. Although originally we planned only to determine the effect of COX-2 inhibitors on the local I/R injury of the intestine, a collaboration with the group of Prof. Peter Ferdinandy allowed us to analyse the intestinal responses to remote (cardiac) I/R injury as well.

a) We have optimized the I/R injury protocol by occluding and releasing the superior mesenteric artery for different time intervals. Based on the myeloperoxidase (MPO) content of intestinal tissue we have chosen 30 min occlusion and 2 h reperfusion periods, and used this surgical protocol in celecoxib- and rofecoxib-treated rats. We found that chronic treatment with celecoxib reduced the severity of I/R-induced inflammation (tissue levels of MPO and cytokines) in a dose-dependent manner. Rofecoxib treatment also tended to ameliorate the

intestinal damage, although the results were less consistent. From these results we concluded that **COX-2 inhibitors protect against intestinal I/R injury**. However, experiments are still going on to explain the differences between the two coxibs.

This work was presented at the PhD Scientific Days 2021 by my PhD student (Szilvia László) (https://phd.kmcongress.com/osszefoglalo/7770), and was also the topic of a diploma work (Tamás Hegyes, Pázmány Péter Catholic University, 2020).

b) We also showed that chronic treatment with rofecoxib inhibited the intestinal damage caused by cardiac I/R. The protective effect of rofecoxib was associated with lower intestinal levels of COX-2 and plasma levels of matrix metalloproteinase-2 (MMP-2). These results suggest that **COX-2 inhibition may also protect against remote intestinal I/R injury**. Interestingly, cardiac ischemic-preconditioning was ineffective, suggesting that different pharmacological or surgical strategies aiming to reduce the extent of cardiac I/R injury do not necessarily provide protection against remote I/R injury arising in distant organs.

László SB et al. Chronic treatment with rofecoxib but not ischemic preconditioning of the myocardium ameliorates early intestinal damage following cardiac ischemia/reperfusion injury in rats. Biochem Pharmacol. 2020 178:114099. IF.: 5.858

2.3. 3rd subproject – Analysing the effect of alpha2-adrenoceptor agonists and other gastroprotective agents on NSAID enteropathy

In this subproject we focused on potential therapies to limit NSAID-induced intestinal damage. Our first candidates were the alpha2-adrenoceptor agonists, because previously we demonstrated that pharmacological stimulation of alpha2-adrenoceptors with clonidine and other drugs is gastroprotective [14,20-22]. Our hypothesis was also supported by a pilot study showing that clonidine reduced the macroscopic signs of indomethacin-induced enteropathy.

a) Unfortunately, we could not reproduce the results of our pilot experiment. Clonidine, given twice daily at different doses, had no significant effect on the severity of indomethacin-induced intestinal damage (macroscopic signs of enteropathy, histology, tissue levels of MPO and interleukin 1β). Hematological analysis (blood cell counts, parameters related to hepatic and renal functions) led to similar results. Another study with another alpha2-adrenoceptor agonist is still ongoing, but at present **our results suggest that pharmacological stimulation of alpha2-adrenoceptors cannot prevent the development of NSAID-enteropathy**. This may be due to the different pathogenesis of gastro- and enteropathy. For example, clonidine-induced gastroprotection is mediated partly by prostaglandins [22], which do not likely have a major protective role in enteropathy [23].

These results were presented at the PhD Scientific Days 2021 by my PhD student (András Tóth) (https://phd.kmcongress.com/osszefoglalo/7854).

b) An unforeseen opportunity to collaborate with Prof. Zoltán Benyó and to work with lysophosphatidic acid 2 (LPA_2) receptor knock out mice prompted us to initiate a study, which was originally not planned. The role of these receptors in the pathogenesis of gastrointestinal inflammatory disorders is controversial and to date no studies have been conducted to determine their effect on NSAID enteropathy. We found that genetic blockade of LPA_2 receptor ameliorates, whereas pharmacological activation aggravates indomethacin-induced intestinal

damage. These results suggest that **LPA_2 receptors may be potential targets in the therapy of NSAID enteropathy**.

These results were be presented in the 4th plenary session of the 62th Annual Meeting of the Hungarian Society of Gastroenterology by my PhD student (Barbara Hutka), and at both the Semmelweis and National Conferences of Scientific Students' Associations by Anett Várallyay (awarded with 1st and 2nd prizes, respectively). We are planning to complete the study and submit a paper this year.

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