MOLECULAR BACKGROUND OF NSAID INDUCED RENAL AND INTESTINAL DAMAGE

PhD Thesis

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1. INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medications worldwide for pain and inflammatory conditions. Approximately 30 million people use NSAIDs daily, with ibuprofen, diclofenac, and naproxen accounting for most of the consumption. Their pharmacological effects occur primarily through inhibition of cyclooxygenases (COX-1 and COX-2), which convert arachidonic acid into prostaglandins. While effective and generally safe for short-term use, high doses or prolonged administration lead to clinically significant adverse events in the kidney and small intestine.

In the kidney, repeated NSAID exposure causes hemodynamic acute kidney injury as well as chronic tubulointerstitial damage that progresses to irreversible fibrosis — the final common pathway of chronic kidney disease. Proximal tubular epithelial cells are particularly vulnerable due to their high metabolic demand. Key mechanisms include TGF-β signaling (the master regulator of fibrosis), EGR1 transcription factor activation, oxidative stress from reactive oxygen species overproduction, and autophagy dysfunction.

Concurrently, NSAID-induced enteropathy affects 50–70% of chronic users and often remains asymptomatic in early stages. This condition involves mucosal injury, barrier disruption, and dysbiosis. Antimicrobial peptides (AMPs) such as cathelicidin and defensins, which maintain intestinal homeostasis, show altered expression during injury, while

systemic hematological changes reflect the broader inflammatory impact. Despite the high prevalence of these complications, the precise COX-independent molecular mechanisms driving NSAID-induced renal fibrosis and enteropathy remain incompletely understood. Current treatments are limited, highlighting the need for deeper mechanistic insights.

Therefore, the present thesis aimed to elucidate the molecular background of NSAID-induced renal and intestinal damage using integrated in vitro and in vivo models, with particular focus on autophagy impairment, oxidative stress, EGR1-mediated profibrotic signaling, and dysregulation of intestinal antimicrobial peptides.

2. OBJECTIVES

The main objectives of this thesis were:

1. To identify the optimal cell culture medium for reproducible TGF-β1-induced epithelial-to-mesenchymal transition and fibrotic responses in HK-2 cells.
2. To examine the dose-dependent effects of indomethacin, celecoxib, and naproxen on cytotoxicity, fibrosis-related genes, autophagy markers, oxidative stress pathways, and EGR1 expression in renal tubular epithelial cells (in vitro) and rat kidney medulla (in vivo).
3. To investigate the structural and molecular changes in the kidney following prolonged NSAID administration, focusing on tubular injury, fibrosis, and autophagy impairment.

4. To determine the time- and dose-dependent effects of indomethacin on antimicrobial peptide expression (cathelicidin, α-defensin 5, β-defensin 2) and systemic hematological parameters in a rat model of NSAID-induced enteropathy.

5. To elucidate the COX-independent mechanisms underlying NSAID toxicity and evaluate the safety profile of selected treatments regarding motor function and gastrointestinal transit.

3. METHODS

HK-2 Cell Culture in Different Media with TGF-β1 Treatment: HK-2 cells were cultured in four different growth media. Cells were seeded in 6-well or 24-well plates and incubated overnight. After serum starvation (except cells in Keratinocyte Serum-Free Medium), cells were cultured in DMEM/F-12 containing 0.5% Fetal Bovine Serum and treated with Transforming Growth Factor Beta 1 or vehicle for 24 hours. Finally, cells were harvested for RNA isolation using TRIzol Reagent.

Treatment of HK-2 and mIMCD-3 cells with NSAIDs: HK-2 and mIMCD-3 cells were seeded in 24-well plates and, after medium change to serum-free DMEM, treated for 24 h with indomethacin (20 µg/mL, reference), celecoxib (0.72–8.7 µg/mL), or naproxen (15–110 µg/mL), all dissolved in 0.4% DMSO. Vehicle controls received DMSO alone. Concentrations were chosen according to reported human peak plasma levels (Cmax) to ensure therapeutic relevance while avoiding excessive cytotoxicity (n=6 per group).

In vivo experimental design for NSAID-induced renal damage:

Male Wistar rats were randomly assigned to six groups (n=8 per group) and received oral gavage twice daily for 14 days. Treatments included celecoxib (10 or 30 mg/kg), naproxen (10 or 20 mg/kg), indomethacin (2 mg/kg, reference COX inhibitor), or vehicle (1% hydroxyethylcellulose). Doses were selected based on human equivalent doses calculated by the Reagan-Shaw formula. The high-dose naproxen group (20 mg/kg) showed severe health deterioration, leading to early euthanasia on day 8. At the study endpoint, animals were euthanized by CO_2 asphyxiation. The left kidney was fixed in 4% buffered formalin for histology, while the right kidney was dissected into cortex and medulla, minced, and snap-frozen in liquid nitrogen for molecular analyses.

In vivo experimental design for NSAID-induced enteropathy:

Two sequential studies were conducted in male Wistar rats to assess indomethacin effects on intestinal antimicrobial peptides. Animals were co-housed to standardize gut microbiota and randomly assigned to vehicle (1% hydroxyethylcellulose) or indomethacin groups.

In the acute study, a single oral dose of 20 mg/kg indomethacin was administered, and rats (n=8 per time point) were sacrificed at 6, 12, 24, 48, and 72 h post-treatment (vehicle controls at 72 h only).

In the chronic study, indomethacin was given at 2 mg/kg twice daily for 14 days or 4 mg/kg twice daily for 7 days. The higher dose caused severe enteropathy, requiring early euthanasia. At study endpoints, blood was collected and the small intestine was removed, snap-frozen in liquid

nitrogen, and stored at –80 °C for molecular analysis.

Hematological analysis: Whole-blood samples were collected in Vacuette K3 EDTA tubes and analyzed for hematological parameters using a Sysmex XN-1000 hematological analyzer according to standard laboratory procedures. Data was automatically calculated by instrument software

RNA Isolation and Quantitative RT-PCR Analysis: RNA was reverse transcribed using specific kits, and PCR reactions were performed using the SensiFast SYBR Green PCR Master Mix. The target gene expression was normalized to the appropriate housekeeping gene and expressed relative to a control sample.

Immunoblot: Proteins were extracted from cells, loaded on gels, and transferred to membranes. The membranes were incubated with primary and secondary antibodies and visualized using a chemiluminescence detection kit. Data was evaluated using Image Studio Lite 5.2 software.

Immunocytochemistry: HK-2 cells were plated in 8-well chamber slides and treated for 24 h with indomethacin (40 µg/mL), celecoxib (2.1 or 8.7 µg/mL), naproxen (30 or 110 µg/mL), vehicle, or TGF-β (10 ng/mL, positive control). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and blocked with 2% donkey serum. Primary antibodies (anti-EGR1 and anti-α-SMA) were incubated overnight at 4 °C, followed by Alexa Fluor 594 and 488 secondary antibodies. Slides were mounted with ProLong Gold containing DAPI and imaged by fluorescence microscopy.

Renal Histology and Immunohistochemistry: Kidney tissues were fixed in formalin and embedded in paraffin. Tubulointerstitial fibrosis and damage were evaluated on Masson's trichrome-stained sections using a blinded semi-quantitative scoring system at 100× magnification. H&E-stained sections were scored (0–4) for tubular dilation, atrophy, and interstitial mononuclear cell infiltration.

For EGR1 immunohistochemistry, heat-induced antigen retrieval (pH 6.0) was performed, followed by blocking with 5% normal goat serum. Sections were incubated overnight at 4 °C with anti-EGR1 primary antibody, then with biotinylated goat anti-rabbit secondary antibody, alkaline phosphatase-streptavidin conjugate, and Vulcan Fast Red chromogen. Staining intensity was graded semi-quantitatively (0–4) at 400×, and EGR1-positive nuclei were counted in multiple high-power fields.

Statistical analysis: Experimental data are presented as mean ± SD. Statistical analysis was performed using IBM SPSS Statistics version 28.0. Normality was assessed with the Shapiro-Wilk test. Depending on the experiment and data type, comparisons were made using one-way ANOVA followed by Holm–Sidak post-hoc test, Kruskal-Wallis test with Dunn’s post-hoc test, Mann-Whitney U test, or Spearman/Pearson correlation. Benjamini–Hochberg correction was applied for multiple comparisons. Statistical significance was set at $p < 0.05$.

4. RESULTS

NSAID-Induced Renal Damage

Optimal cell culture medium for HK-2 cells: TGF-β1-induced EMT was compared in four media. TGFB1 mRNA increased in all, but EGR1 correlation was strongest in DMEM 5% FBS, where TIMP1 and LGALS3 were also significantly upregulated. This medium was selected as optimal for subsequent NSAID-induced renal fibrosis studies.

Induction of fibrosis-related pathways and autophagy dysregulation: High-dose celecoxib and naproxen (but not indomethacin) caused marked dose-dependent upregulation of fibrotic genes (TGFB1, ACTA2/α-SMA, TIMP1, COL1A1, LCN2), AP-1 components (JUN, FOS), and EGR1/EGR2 in both HK-2 and IMCD cells. Autophagy-related genes (BECN1, ATG7, LC3A) also increased. At the protein level, the LC3-II/I ratio rose while p62 accumulated, indicating impaired autophagic flux.

Renal tubular dilation and atrophy in rats: Prolonged high-dose celecoxib (30 mg/kg) and naproxen (20 mg/kg) induced mild but statistically significant tubular dilatation, atrophy, and reduced epithelial cell density compared to vehicle controls. Mononuclear cell infiltration showed only a slight, non-significant increase.

Dose-dependent activation of renal fibrotic pathways: High-dose celecoxib and naproxen strongly upregulated mRNA levels of Tgfb1, Timp1, Col1a1, Egr1, and the tubular injury marker Lcn2 in the renal medulla. AP-1 components (cFos, cJun) were also markedly increased. Naproxen produced stronger effects than celecoxib, and even low-dose

naproxen (10 mg/kg) significantly elevated these mediators. These changes were consistent with the in vitro findings in HK-2 and IMCD cells.

Autophagy impairment and oxidative stress in renal medulla: High-dose celecoxib and naproxen upregulated autophagy genes (Atg7, Becn1), increased the LC3-II/LC3-I ratio, and caused p62 accumulation, indicating blocked autophagic flux. Simultaneously, Hmox1 and Hif1a mRNA, HMOX1 protein, and pAKT levels rose markedly, confirming activation of oxidative stress and hypoxia-response pathways.

NSAID-Induced Enteropathy

Indomethacin-induced changes in intestinal antimicrobial peptides and hematology parameters: Acute indomethacin (20 mg/kg) caused time-dependent upregulation of cathelicidin (Camp) mRNA from 12 h post-treatment, with positive correlation to inflammatory genes (IL1B and TNF). α-Defensin 5 (Defa5) and β-defensin 2 (Defb2) showed high variability without significant changes. In chronic administration, cathelicidin and α-defensin 5 were upregulated (stronger at higher dose), while hematological parameters revealed reduced red blood cell indices and increased platelet counts.

5. CONCLUSIONS

1. DMEM s5% FBS was identified as the optimal and most reproducible culture medium for TGF-β1-induced EMT and EGR1 activation in HK-2 cells, solving a major source of variability in renal fibrosis models.
2. High-dose celecoxib and naproxen induce renal tubular injury and fibrosis through COX-independent mechanisms, including autophagy impairment, oxidative stress, and EGR1-mediated profibrotic signaling.
3. These effects were consistently observed in both HK-2/IMCD cells in vitro and rat renal medulla in vivo.
4. Indomethacin triggers rapid, inflammation-driven upregulation of cathelicidin (Camp) in the small intestine, with dose- and time-dependent changes in α-defensin 5.
5. Systemic hematological alterations (reduced RBC indices and increased platelets) serve as reliable markers of enteropathy severity.
6. Autophagy dysfunction, oxidative stress, and EGR1 activation are central mediators of NSAID nephrotoxicity.
7. These findings highlight new therapeutic targets: autophagy rescue, EGR1 inhibition, and modulation of antimicrobial peptides to reduce NSAID-induced renal and intestinal damage.

6. PUBLICATIONS

Publications related to the thesis:

1. **Haghighi S**, Haghighi A, Zádori ZS, Kovács K, Manzéger A, Kökény G. Celecoxib and naproxen disrupt autophagy and activate EGR1 in kidney tubules. Experimental and Molecular Pathology. 2025;144:105000. Impact factor, journal quartile – 3.7, Q1.

2. Garmaa G, Manzéger A, **Haghighi S**, Kökény G. HK-2 cell response to TGF-β highly depends on cell culture medium formulations. Histochemistry and Cell Biology. 2024;161(1):69-79. Impact factor, journal quartile – 2.3, Q1.

3. Haghighi A, Tóth AS, Demeter ZO, Hutka B, Zsidai A, Lengyel L, **Haghighi S**, Pannier M, Le Cosquer G, Meunier ES, Ágg B, Makra N, Ostorházi E, Ligeti B, Kovács K, Kelemen Á, Jakab A, Wachtl G, Kökény G, Szabó D, Zádori ZS. Oral indomethacin modifies small intestine biofilms and host-microbe interaction mediators. Life Sciences. 2025;384:124114. Impact factor, journal quartile – 5.1, D1.

Publications not related to the thesis:

1. Haghighi Bardineh SA, Balou HA, Sedigh Ebrahim-Saraie H, Mobayen M, Esmailzadeh M, **Haghighi S**, Haghighi A, Sadeghi M. Predictive value of serum albumin and calcium levels in burn patients with Pseudomonas aeruginosa infection: A comprehensive analysis of clinical outcomes. International

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2. Salehi Z, Haghighi A, **Haghighi S**, Aminian K, Asl SF, Mashayekhi F. Mitochondrial DNA Deletion Δ4977 in Peptic Ulcer Disease. Molecular Biology. 2017;51(1):30-33. Impact factor, journal quartile – 0.977, Q3.