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ORIGINAL ARTICLE

Effects of Mesalazine on Morphological and Functional Changes in the Indomethacin-Induced Inflammatory Bowel Disease (Rat Model of Crohn's Disease)

Higin Simon[1] · **Tamás Fischer**[1] · **Attila Almási**[2] · **Emil Fischer**[1]

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Abstract Morphological and functional changes have been investigated in the rat model of Crohn's disease. The inflammatory bowel disease was induced by indomethacin (1 × 10 mg/kg s.c. for 3 days). Morphological alterations were evaluated by macroscopic scoring system and on the base of histological changes in the small intestine. Functional activities were studied by determination of the intestinal and hepatic elimination of p-Nitrophenol (PNP) and its metabolites (PNP-glucuronide: PNP-G and PNP-sulfate: PNP-S) during the luminal perfusion of PNP. It was found that the indomethacin induced severe macroscopic changes (hyperaemia, petechia, bleeding, erosions, ulcerations) and significant histological alterations in the small intestine of rats which were definitely inhibited by mesalazine (1000 mg/kg by gastric tube for 3 days). Disappearance of PNP from the luminal perfusion solution was diminished by indomethacin which was corrected by administration of mesalazine. Significant depression was found in the luminal appearance of PNP metabolites by giving of indomethacin and these alterations could not be compensated by mesalazine.

Hepatic elimination of PNP (biliary excretion of PNP and its metabolites) was decreased definitely by indomethacin which was – at least partly - compensated by mesalazine.

The findings of the present study suggest that the indomethacin-induced inflammation in the small intestine represents a useful rat model of Crohn's disease. Morphological and functional alterations caused by indomethacin can be compensated by mesalazine.

Keywords Inflammatory bowel disease · Crohn's disease · Indomethacin · Mesalazine · p-Nitrophenol

Introduction

It is known that indomethacin can produce inflammatory reactions in the small intestine in experimental animals [1–3]. Indomethacin is a non-selective cyclooxigenase inhibitor and decreases the prostanoid formation, furthermore it has some other effects too, e.g. on the oxidative phosphorylation and on the structure and the function of epithelial cell membrane and the brush border [4, 5]. Different factors (e.g. drug administration including antibiotics and non-steroidal antiinflammatory drugs (NSAIDs), changes in the food uptake and in the biliary flow) can influence the indomethacin-induced inflammatory bowel disease [6–9]. It has been published that there are similarities between ileal Crohn's disease and indomethacin-induced experimental jejunal ulcer in the rat [10]. It is interesting that there is an increased intestinal permeability in Crohn's disease, while NSAIDs, including indomethacin can also enhance the intestinal permeability in rats [11]. The above mentioned alterations are important e.g. in some other functions and they can also influence the drug elmination including first of all the drug metabolism and excretion in the intestinal tract and in the liver. Therefore the aim of our present investigations was to study the morphological changes and some functional alterations, namely the intestinal and hepatic drug metabolism and excretion in the indomethacin-induced inflammatory bowel disease. For the investigation of functional and pharmacological changes a middle segment of small intestine of rats was perfused with

✉ Emil Fischer
emil.fischer@aok.pte.hu

[1] Department of Pharmacology and Pharmacotherapy, Medical Faculty, University of Pécs, 12 Szigeti út, Pécs, Hungary

[2] Department of Pharmaceutical Chemistry, Pharmaceutical Faculty, University of Pécs, 2 Rókus utca, Pécs, Hungary

isotonic medium containing PNP in a concentration of 500 μM, this experimental arrangement was basically similar to the oral drug administration. Bile duct of rats was cannulated and the bile was collected for the investigation of hepatic elimination function. Samples were obtained from the luminal perfusion solution and from the bile and the concentration of p-Nitrophenol (PNP) and its metabolites was measured which allowed to determine the elimination function of the small intestine and the liver. PNP was used as a model compound because many drugs contain phenolic structure and hydroxyl groups, therefore the metabolism and excretion of these drugs are similar to those of PNP. Moreover, it is well known that PNP is metabolized almost exclusively by conjugation and forms two metabolites: p-nitrophenol-glucuronide (PNP-G) and p-nitrophenol-sulfate (PNP-S) [12–14]. There are different HPLC methods for the analysis of these metabolites of PNP, which were modified in our previous experiments [15].

Materials and Methods

Materials, Chemicals for Analysis

p-nitrophenol, its glucuronide, the monopotassium salt of p-nitrophenol were obtained from the Sigma Aldrich Company (Budapest, Hungary). All other chemicals and reagents were analytical or HPLC grade. The standard isotonic perfusion solution had the following compositions (mmol/l): NaCl 96.4, KCl 7.0, $CaCl_2$ 3.0, $MgSO_4$ 1.0, sodium phosphate buffer (pH 7.4) 0.9, TRIS buffer 29.5, glucose 14.0, mannitol 14.0.

Animals and Experimental Procedure

Male Wistar rats weighing 220–250 g were used. The animals were anesthetized with urethane (1.2 g/kg i.p.). The abdomen was opened by a mid-line incision and a middle segment (length about 10 cm) of small intestine was cannulated in vivo. The lumen was gently flushed with warm (37 °C) isotonic solution to remove digesta and food residues and then blown empty with 4–5 ml air. Perfusion through the lumen of cannulated segments of small intestine with isotonic medium containing PNP was carried out at rate of 13 ml/min in a recirculation mode for 90 min. In control rats the lumen of intestinal segments was perfused with isotonic medium without PNP. The volume of samples obtained from the perfusion medium coming out from the cannulated segments was 250 μl, the initial perfusion volume was 15 ml. The temperature of perfusion medium was maintained constant at 37 °C. The animals were fasted 16–20 h prior to the experiments, water was given ad libitum.

For the investigation of biliary flow and biliary excretion of PNP and its metabolites the bile duct was cannulated with a polyethylene tube (PE-10) and the bile was collected in 15 min-periods.

Inflammatory bowel disease (model of Crohn's disease) was induced by indomethacin (1 × 10 mg/kg subcutaneously on day 1, day 2 and day 3). Mesalazine was administered (1 × 1000 mg/kg by gastric tube) at day 1, day 2 and day 3. Experiments were carried out at day 4. The evaluation of severity of macroscopic changes was performed by scoring system (Table 1). For the microscopic analysis the sections of the segments of small intestine were routinely fixed and stained by hematoxylin eosin.

Analytical Conditions, Instrumentation, Determination

The samples obtained from the intestinal perfusion medium and from the bile were analyzed and quantified by the HPLC methods which have developed and used in our related experiments [15–17]. Summerized shortly: the mobil phase consisted of methanol and distilled water (50:50 *v*/v%) containing 0.01 M tetrabutyl ammonium bromide to determine the metabolites. The samples were vortexed and centrifuged at 3000 g for 10 min before separation. The flow rate of the eluent was 1.2 ml/min. The volume of the samples was 20 μl and the detection was effected at 290 nm, because this wavelength was optimal for the simultaneous determination of PNP, PNP-G and PNP-S. At the bile samples the mobil phase was consisted of methanol and citrate buffer pH 6.2 (47:53 *v*/v%) containing 0.03 M tetrabutyl ammonium bromide with a

Table 1 Scoring system for the evaluation of severity of indomethacin-induced bowel inflammation

Macroscopic score	Definition
0 point	No change in the mucosal and serosal area
1 point	Hyperaemia and/or petechia
2 points	One mucosal erosion or ulceration
3 points	One mucosal erosion or ulceration with hyeremia, serosal and mesenterial adherences or bleeding
4 points	Multiplex erosions or ulceration (extensions less than 10 cm)
5 points	Multiplex erosions/ulcerations (extensions graeter than 10 cm)

The scoring system was determined on the effects of defferent doses of indomethacin form 1 × 7.5 to 16 mg/kg s.c. for 3 days. Investigations were carried out on day 4

flow rate of 1.0 ml/min. The samples were prepared by mixing 200 μl cold methanol containing 3.125 M p-ethylphenol as internal standard, and 50 μl bile. After vortexing, the samples were centrifuged by 10,000 g for 10 min, and 20 μl of supernatants were injected to the HPLC.

The samples were stored at −20 °C and before the analysis the tempereture of the samples was allowed to rise to ambient temperature.

The HPLC system consisted of a Varian 2010 pump, a Rheodyne 7725i injection valve, an UV-detector 308 with a PowerChrom 280 data collecting and integrating unit and software.

A Nucleosil 100 C_{18} reversed phase column (250 mm × 4.6 mm ID, 10 μm particle size) was employed for the separation of metabolites, while at the bile samples Teknokroma TR-C-160K1 guard column was applied also.

Calculations, Statistical Analysis

The luminal appearances of PNP-G and PNP-S were calculated on the base of their luminal concentrations and the actual volume of perfusion solution. The original volume of perfusion medium was 15 ml which was modified by the obtained samples and the absorptive and resorptive activity of the perfused intestinal segment. Therefore the actual volume of perfusion solution was calculated and corrected by these changes. The biliary excretion of PNP and its metabolites was calculated as the product of biliary volume and the biliary concentration of compounds.

Data show the mean values ± S.E. (n: number of experiments or determinations).

Data were analyzed by one–way ANOVA, the difference among groups was determined by Student's t-test.

Results

Table 1 demonstrates the scoring system for evaluation of severity of indomethacin-induced inflammatory reactions in the small intestine. The scoring system was validated on the basis of the effect of different indomethacin dosages (from 1 × 7.5 to 16 mg/kg s.c. for 3 days). The severity of bowel inflammation (hyperemia, petechia, bleeding, lesions, ulcerations) was observed and determined at day 4. using different points of the scoring system.

Effects of the mesalazine on the indomethacin-induced bowel inflammation rat model are summarized in Table 2. Indomethacin was given 1 × 10 mg/kg s.c. for 3 days, mesalazine was administered 1 × 1000 mg/kg by gastric tube for 3 days. The investigations were performed at day 4. Indomethacin provoked severe signs of inflammation in the small intestine (3 and 4 points of scoring system). However mesalazine was able to protect definitely the actions of indomethacin, less severe changes (1 and 2 points of the scoring system) were observed in the small intestine after the combined administration of indomethacin and mesalazine.

Table 2 Effect of Mesalazine on the indomethacin-induced bowel inflammation rat model

Macroscopic score	Indomethacin	Indomethacin + Mesalazine
0	0	0
1	0	9
2	0	6
3	4	0
4	11	0
5	0	0

Indomethacine was administered 1 × 10 mg/kg s.c. for 3days, Mesalazine was given gastric tube 1 × 1000 mg/kg for 3 days. Investigations were carried out on day 4

The next three figures (Fig. 1, Fig. 2. and Fig 3.) present the microscopic pictures of the control (Fig.1.) and ulcerated bowel walls (Fig. 2. and Fig. 3.) It can be seen that indomethacin (given 1 × 10 mg/kg s.c. for 3 days, investigation at day 4) induced acute ulcer in the small intestine. The ulcer was deep, even the inner isoproprial muscular layer was involved and a nonspecific inflammation could be seen in the fat tissue beneath (Fig.2.). Mesalazine produced a protective action and Fig. 3. shows that ulcer was less deep, the two thirds of the inner muscular layer was preserved after the combined administration of indomethacin and mesalazine.

Functional changes and alterations of the metabolizing and excretory capacity of the small intestine and liver are demonstrated in the next Figures. Disappearance of PNP from luminal perfusion solution (containing PNP in a concentration of 500 μM) in control and indomethacin-pretreated rats is shown in Fig. 4. As it can be seen, from the 30 min indomethacin produced significantly higher PNP levels in the luminal

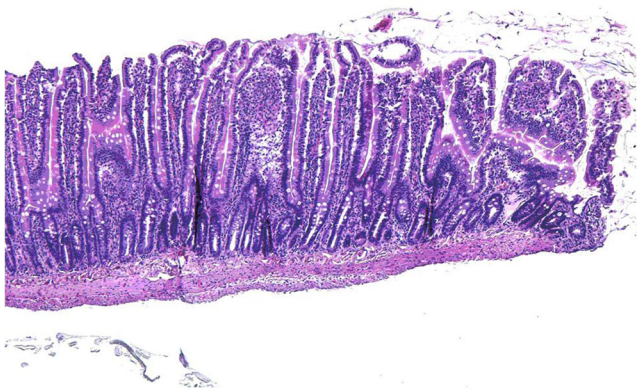

Fig. 1 Microscopic picture of normal (non-ulcerated) small bowel of the control rat. Hemotoxylin-eosin staining, ×40

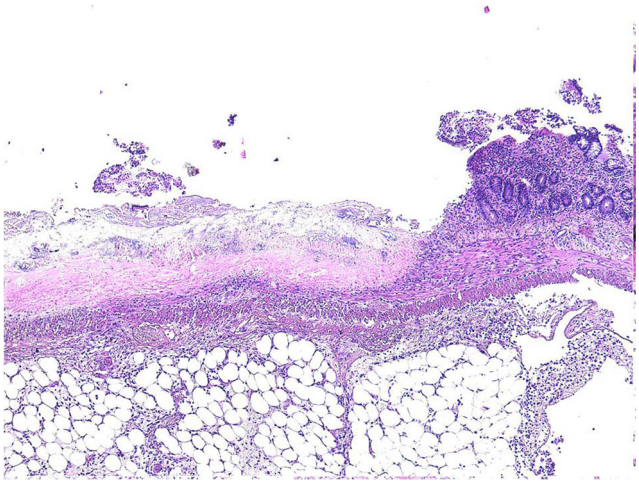

Fig. 2 Low-power micrograph of indomethacin induced (1 × 10 mg/kg s.c. for 3 days) acute ulcer in the small bowel of the rat. The ulcer is deep, even the inner isoproprial muscular (IM) layer is completely involved. Nonspecific inflammation can be seen in the fat tissue beneath. Hemotoxylin-eosin staining, ×40

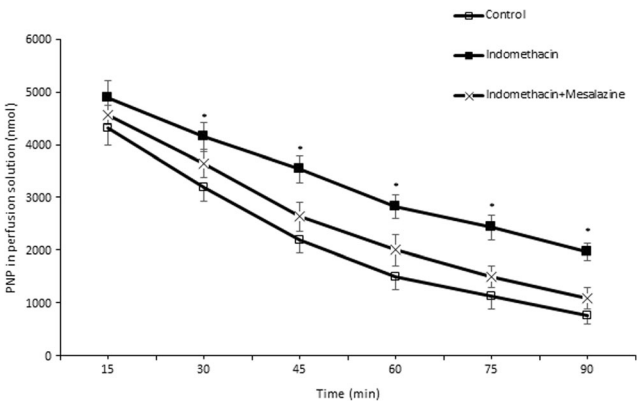

Fig. 4 Disappearance of PNP from luminal perfusion solution in control (□), indomethacin-pretreated (■) and indomethacin + mesalazine pretreated (×) rats. Indomethacin pretreatment: 1 × 10 mg/kg s.c. for 3 days; mesalazine pretreatment: 1 × 1000 mg/kg by gastric tube for 3 days. The middle segment of the small intestine of rats was perfused with isotonic solution containing PNP in a concentration of 500 μM. Data represent the mean value ± S.E. of 9 rats. Significant differences from the control values: * $p < 0.05$

perfusion solution, which means a slower disappearance (absorption) rate or an enhanced blood bood to lumen flux of PNP due to an increased permeability of the small intestine in this direction. It is interesting to note that mesalazine corrected the effect of indomethacin and the values of PNP disappearance increased after the combined administration of indomethacin and mesalazine, these data show no significant differences from those of controls.

Cumulative (measured for 90 min) luminal apperanace of PNP-metabolites (PNP-G and PNP-S) is demonstrated in Fig.5. Indomethacin depressed definitely the luminal appearance of PNP-G and PNP-S, however this decreasing effect of indomethacin was not influenced significantly by mesalalzine, that is the depressed luminal appearance of the metabolites was not stimulated by simulataneous administration of mesalazine and indomethacin.

The values of hepatic elimination (biliary excretion) of PNP are summarized in Fig. 6. The results of these experiments indicate that both the PNP and its metabolites (PNP-G and PNP-S) are appeared in the bile during the intestinal perfusion of PNP. Indomethacin inhibited significantly the biliary excretion of PNP and its metabolites (PNP-G and PNP-S), as well. Mesalazine produced a protective action in all cases, that is, it stimulated definitely the indomethacin-depressed biliary excretion rate of PNP, PNP-G and PNP-S, however the control level of biliary excretion was reached only in the case of PNP-S.

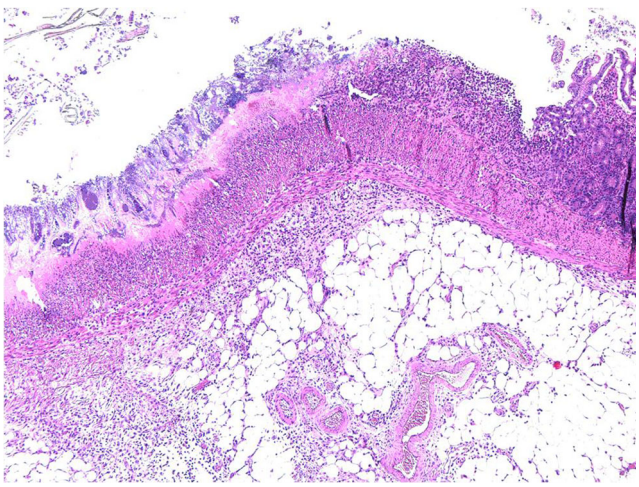

Fig. 3 Low-power micrograph of acute ulcer in the small intestine of the rat. Ulcer was produced by indomethacin (1 × 10 mg/kg s.c. for 3 days) and the rats were treated by mesalazine (1 × 1000 mg/kg by gastric tube for 3 days). Superficial necrotic layer (N) can be seen with basophilic bacterial clouds with the inflammatory zone beneath. The ulcer is less deep, the lower two thirds of the inner muscular layer is preserved. Hemotoxylin-eosin staining, ×40

Discussion

In these experiments the morphological and functional (pharmacological) changes were studied in the indomethacin-induced inflammatory bowel disease (rat model of Crohn's disease). Indomethacin was administered in a similar protocol which was published by other authors [3, 18–20]. In our experimental arrangement 10 mg/kg/s.c. 1 x daily for three days) proved to be optimal for the investigation of morphological and functional changes provoked by indomethacin.

It was found that indomethacin produced characteristic morphological (macroscopic and microscopic) alterations in the small intestine which are basically similar to those of Crohn's disease [10]. Mesalazine was able to produce protective actions, that is, less severe changes were detected using the scoring system and analyzed on the base of microscopic pictures. These results indicate that the experimental

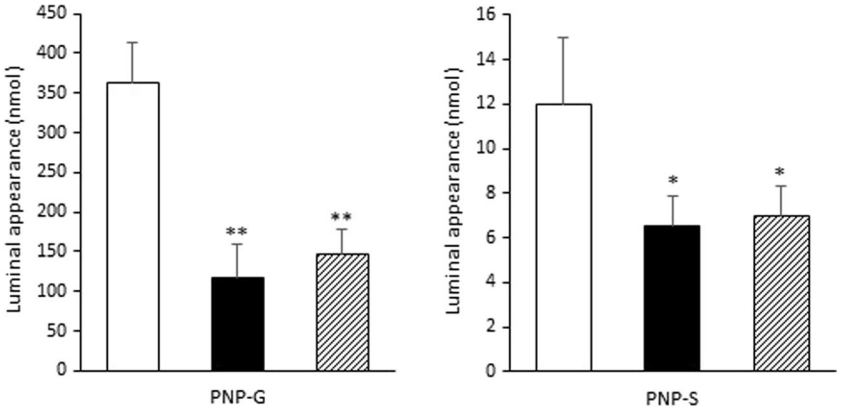

Fig. 5 Cumulative luminal appearance of PNP-G and PNP-S in perfusion solution in control (□) indomethacin pretreated (■) and indomethacin + mesalazine pretreated (▨) rats. Indomethacin pretreatment: 1 × 10 mg/kg s.c. for 3 days; mesalazine pretreatment: 1 × 1000 mg/kg by gastric tube for 3 days. The middle segment of the small intestine of rats was perfused with isotonic solution containing PNP in a concentration of 500 μM. Data represent the mean value ± S.E. of 9 rats. Significant differences from the control values: * $p < 0.05$, ** $p < 0.01$. No significant differences were found between the values of indomethacin and indomethacin + mesalazine pretreated rats

arrangement used in our investigations proved to be a good tool for induction of inflammatory bowel disease as a rat model of Crohn's disease. Moreover, this model is also suitable to analyze the protective actions of drugs, e.g. mesalazine, which is a non-steroidal antiinflammatory agent.

The functional/pharmacological changes in inflammatory bowel disease were also investigated in our experiments. The disappearance of xenobiotics (e.g. PNP) from the intestinal lumen depends first of all on the permeability (absorption) and the elimination processess (metabolism and excretion) in the intestinal tract.

We have found that the disappearance of PNP from the intestinal lumen was decreased by indomethacin, because greater amount of PNP remained in the luminal perfusion solution in indomethacin-pretreated rats than in controls. The intestinal elimination of PNP (luminal apperance of PNP-metabolites) was decreased, however, this depression can not explain the definitely greater difference in the amount of disappearance of PNP from the luminal solution. Therefore our results suggest a smaller absorption rate or a greater blood to lumen flux of PNP in the indomethacin-pretreated rats. Other autors [3, 9, 21, 22] have found also changes in the intestinal permeability in Crohn's disease or due to the action of indomethacin which means usually an increased lumen to blood flux. However, our results suggest an enhanced blood to lumen permeability, that is a stimulated flux of PNP from blood into the intestinal lumen. It is interesting that the depressing effect of indomethacin in the luminal appearance of PNP-G and PNP-S could not be protected by mesalazine, whereas the changes of disappearance of PNP from the luminal perfusion solution in the indomethacin-pretreated rats was compensated by administration of mesalazine. These results suggest that the indomethacine-induced changes in the intestnal drug metabolism and permeability were not

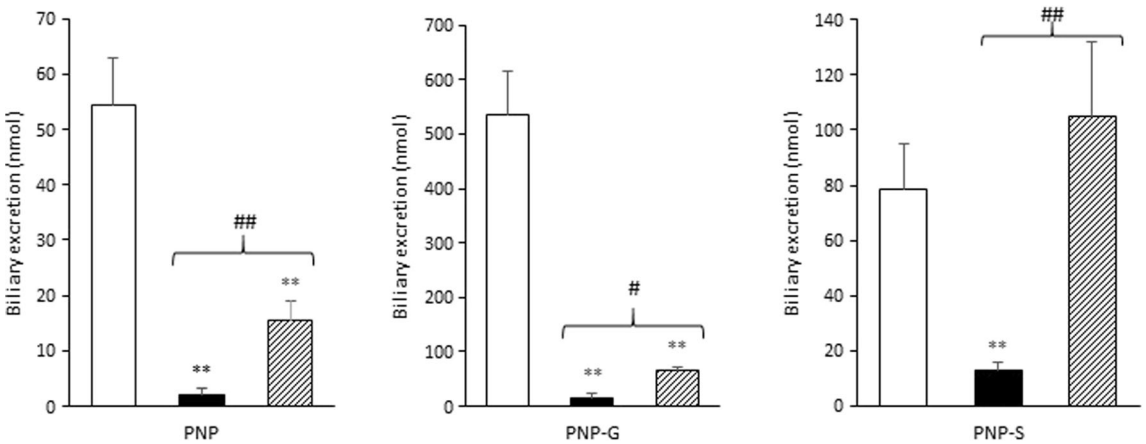

Fig. 6 Cumulative biliary excretion of PNP, PNP-G and PNP-S in perfusion solution in control (□) indomethacin pretreated (■) and indomethacin + mesalazine pretreated (▨) rats. Indomethacin pretreatment: 1 × 10 mg/kg s.c. for 3 days; mesalazine pretreatment: 1 × 1000 mg/kg by gastric tube for 3 days. The middle segment of the small intestine of rats was perfused with isotonic solution containing PNP in a concentration of 500 μM. Data represent the mean value ± S.E. of 9 rats. Significant differences from the control values: ** $p < 0.01$. Significant differences between the values of indomethacin and indomethacin + mesalazin pretreated rats: # $p < 0.05$, ## $p < 0.01$

influenced uniformly, that is mesalazine can produce independent effects on the intestnal elimination of PNP and on the intestinal permeability.

The hepatic elimination of PNP metabolites (PNP-G, PNP-S) was considerably inhibited by indomethacin similarly to the luminal appearance of PNP-G and PNP-S. However, the depressed biliary excretion of PNP-G and PNP-S was compensated by mesalazine which means a sharp contrast with the intestinal elimination of this metabolites. Moreover, the biliary excretion of the mother compound (PNP) in non-metabolized form was also diminished by indomethacin, which means that indomethacin was able to influence not only the metabolism of PNP, however, the biliary excretion of the mother compound (PNP), as well. Yamada et al. (8) found also a negative effect of indomethacin on the bile acid-independent bile flow, and the biliary secretion of bile acids, however, the drug elimination was not investigated in their experiments.

These results show that in the indomethacin-induced bowel inflammation severe changes occur in the intestinal tract, however alterations can be detected in other organs, e.g. in the liver, as well. Furthermore, the changes in the small intestine and in the liver can be partly similar, but differences can be detected, too.

Further investigations are needed to clarify the mechanism of action of indomethacin in the intestinal and hepatic elimination processes and the differences in the protective effects produced by mesalazine.

Compliance with Ethical Standards

Ethical Approval All procedures were carried out on animals according to the Hungarian Animals Act (Scientific Procedures, 1998), and the study was approved by the Ethics Committee on Animal Research of the University of Pécs.

Conflict of Interest The authors declare that they have no conflict of interest.

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