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ORIGINAL COMMUNICATION

Recombinant human lactoferrin ingestion attenuates indomethacin-induced enteropathy *in vivo* in healthy volunteers

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Objective: To determine whether recombinant human lactoferrin ingestion inhibits nonsteroidal antiinflammatory drugs (NSAID)-induced gastroenteropathy *in vivo* in healthy volunteers as a model for disorders associated with a rise in permeability of the stomach and the small intestine.

Design: A randomized crossover dietary intervention.

Subjects and interventions: In all, 15 healthy volunteers (age 23 ± 1.4 y) were tested. A sucrose and a lactulose/rhamnose (L/R) permeability test was performed to assess gastroduodenal and small intestine permeability as indicator of NSAID-induced gastroenteropathy. All subjects consumed standardized meals for 2 days. On the second day at time = -24 h each subject ingested a drink containing 5 g recombinant human lactoferrin or placebo during breakfast. At $t = -9$ h, subjects ingested the same drink with 75 mg of the NSAID indomethacin and after an overnight fast at $t = -1$ h subjects consumed the drink and 50 mg indomethacin. After 1 h, at $t = 0$, a permeability test was performed.

Results: Small intestine permeability after indomethacin and placebo was significantly higher (L/R ratio = 0.036; 0.014–0.092, $P < 0.05$) compared to the permeability observed after ingestion of indomethacin and lactoferrin (0.028; 0.015–0.056), whereas gastroduodenal permeability did not differ between the two interventions ($P = 0.3$).

Conclusion: Oral recombinant human lactoferrin supplementation during a short-term indomethacin challenge reduced the NSAID-mediated increase in small intestinal permeability and hence may provide a nutritional tool in the treatment of hyperpermeability-associated disorders.

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Keywords: lactoferrin; recombinant human lactoferrin; small intestine; permeability; indomethacin; enteropathy

Introduction

In normal conditions, only minute amounts of pathogens permeate the intestinal epithelium. However, if the intestinal permeability is increased, excessive penetration of

luminal aggressive agents and antigens may attract local and circulating neutrophils which cause the release of reactive oxygen species (Bjarnason *et al*, 1993) and the initiation of gastrointestinal inflammation (Wallace, 1993; Soderholm & Perdue, 2001). This inflammation increases permeability to a greater extent, resulting in permeation of luminal aggressive agents through the mucosa and aggravation of local tissue inflammation which, in turn, leads to increased permeability (Ferry *et al*, 1989). An increase in intestinal permeability may finally result in a significant intrusion of pathogens, such as endotoxin, into the circulation and implies a serious health hazard. An increase in intestinal permeability or a decrease in intestinal barrier function is regarded as a pathophysiological factor in Crohn's disease (Meddings, 1997), celiac disease (Smecuol

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Contributors: FJT, WHMS and R-JMB initiated the study and discussed the study design and study outcomes. FJT prepared the experimental protocol, carried out the practical work and prepared the drafts of the paper, WHMS and R-JMB secured the funding, supervised the funding and discussed and revised the drafts of the paper.

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et al, 1997), viral gastroenteritis (Isolauri *et al*, 1989) and food allergy (Crowe & Perdue, 1992). Shortly after the administration of indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), small intestinal permeability is increased in the same order of magnitude as in these disorders (Bjarnason *et al*, 1986). In the present study, we administered indomethacin to healthy volunteers as a model for the gastroenteropathy associated with a rise in small intestinal permeability. Controlling permeability with specific food ingredients such as lactoferrin (LF) may provide a tool in the treatment of these disorders.

In animal studies, several beneficial effects were attributed to LF. Bactericidal (Bellamy *et al*, 1992; Zagulski *et al*, 1998), anti-inflammatory (Nuijens *et al*, 1996), antiviral (Swart *et al*, 1998; Ikeda *et al*, 2000; Murphy *et al*, 2000) and antioxidative activities (Gutteridge *et al*, 1981; Baldwin *et al*, 1984; Davidson & Lonnerdal, 1989) were discovered previously. Concomitant ingestion of lactoferrin together with indomethacin may protect against indomethacin-induced gastroenteropathy. LF is a protein of the transferrin family. It is present in milk, saliva, tears, bile, blood plasma, mucosal and genital secretions (Britigan *et al*, 1994; Levay & Viljoen, 1995) and released by neutrophil-specific granules (Davidson & Lonnerdal, 1989). Since LF is present in relatively high concentrations in human colostrum, it was thought to play an important role in the development of newborns. This hypothesis was strengthened by the discovery of specific LF receptors on the intestinal brush border (Mazurier *et al*, 1985; Kawakami & Lonnerdal, 1991), lymphocytes (Legrand *et al*, 1992) and possibly in hepatocytes (McAbee & Esbensen, 1991). The physiological effects of LF have been reviewed by several investigators (Baker *et al*, 1991; Iyer & Lonnerdal, 1993; Britigan *et al*, 1994; Brock, 1995; Levay & Viljoen, 1995), but the exact mechanisms of action are still unclear. Some of the observed and hypothesized effects of LF may be regulated through binding to a specific LF receptor in the small intestine. However, binding of LF to its receptor is a species-specific process. It was shown previously that bovine LF does not recognize the human LF receptor, whereas rhesus monkey lactoferrin and human LF are structurally and functionally identical and have the same affinity for the intestinal brush-border membrane LF-receptor (Davidson & Lonnerdal, 1986, 1988).

We investigated whether recombinant human LF was able to attenuate NSAID-induced gastroenteropathy and may thus provide a nutritional intervention tool in the prevention or treatment of disorders associated with an increased intestinal permeability.

Subjects and methods

Subjects

In all, 15 healthy nonsmoking male volunteers (age 23.9 ± 2.2 y) were recruited for participation. All subjects were free of any medication and did not have a history of a gastrointestinal disorder. The study was carried out

according to a double-blind randomized crossover design. Each subject participated in two experiments with a wash-out period of 2 weeks in between. All subjects were informed orally and in writing about the aim and the protocol of the study and gave their written informed consent before participation. The study protocol was approved by the Ethics Committee of the University Hospital Maastricht, Maastricht, The Netherlands.

Protocol

To avoid high levels of dietary lactoferrin intake, subjects consumed a provided standardized diet and were not allowed to consume any dairy products on the 2 days prior to the day of testing. For breakfast and lunch bread rolls of any kind and nondairy drinks were allowed *ad libitum*. Dinner consisted of a commercially available prepared pasta meal (Dinner 2 today, Bami Goreng, Honig, Koog a/d Zaan, The Netherlands). On each test day, a sucrose/lactulose/rhamnose test was carried out as a measure of gastroduodenal and small intestinal permeability. At 24 h prior to the permeability measurement, immediately after breakfast, subjects ingested a test drink. The flavored test drink contained either 5 g recombinant human lactoferrin (rhLF, Agennix inc., Houston, TX, USA) dissolved in 100 mL water or flavored placebo (100 mL water), present in a masked flask. In the evening, exactly 9 h prior to the permeability measurement, subjects ingested a test drink and 75 mg indomethacin (Genfarma bv., Maarsse, The Netherlands) and after an overnight fast, 1 h before permeability assessment, subjects ingested a test drink and 50 mg of indomethacin. After 1 h, after voiding, a hyperosmolar drink containing 20 g sucrose, 5 g lactulose (Genfarma bv., Maarsse, The Netherlands) and 0.5 g L-rhamnose (ICN Biomedicals Inc., Aurora, OH, USA) dissolved in 100 ml water was ingested. Subsequently, urine was collected for 5 h. Subjects stayed in the lab and were not allowed to eat during the time of urine collection. Consumption of water was allowed *ad libitum* during the last 2 h of urine collection. The urine volume was determined. Urine aliquots were taken and stored at -80°C until analysis.

At 2 weeks after the first experiment, subjects commenced the same protocol with the complementary test drink.

In an additional study to the effects of rhLF on small intestinal permeability, five of the above subjects ingested a lactulose/rhamnose/sucrose solution as described above after an overnight fast and after voiding, with concomitant ingestion of either 5 g rhLF, dissolved in 100 ml water, or 100 ml water in masked flasks on two separate days. After ingestion of the beverage, volunteers collected urine for 5 h as described above. The experiments were carried out to conform to a randomized crossover design.

Gastrointestinal permeability

Urinary sucrose excretion provides an estimation of the permeability of the stomach and the proximal duodenum (Meddings *et al*, 1993). Urinary sucrose was determined with a Dionex ion chromatography system (Dionex Corporation, Sunnyvale, CA, USA). All samples were spiked with sucrose to enable identification of sucrose in the complex urine matrix. Samples were put on a column (Dionex PA1 and guard column PA1) with a sodium hydroxide—sodium acetic acid gradient. Sucrose was detected using pulsed electrochemical detection.

Intestinal permeability was assessed using the lactulose/rhamnose gut permeability test. This test is based on the comparison of intestinal permeation of molecules of different sizes. The urinary lactulose/rhamnose excretion ratio is considered to be an accurate parameter for small intestinal permeability (van Nieuwenhoven *et al*, 1999). Lactulose and rhamnose were determined by fluorescent detection high-pressure liquid chromatography (HPLC). All samples were spiked with lactose as an external standard that does not interfere with the determination of lactulose and rhamnose. Samples were incubated overnight with derivatization reagent (9-fluorenylmethyl chloroformate hydrazine) at 65°C. After cooling to room temperature the reaction mixture was injected into a dedicated HPLC system. The fluorescence was detected with a fluorescence detector (B&L systems, Zoetermeer, The Netherlands). The protocol for lactulose and rhamnose analysis was well validated and described in detail previously (Rooyackers *et al*, 1996).

In vitro lactoferrin-indomethacin binding study

An *in vitro* binding study to examine the ability of rhLF to bind indomethacin was performed. An aqua solution containing 25 mg/l indomethacin and 2.5 g rhLF was incubated for 7 and 22 min, respectively. Subsequently, 0.5 ml of the rhLF/indomethacin solution was put over a 30 kDa micropore filter and the amount of indomethacin present in the filtrate solution was determined by HPLC.

Statistics

Urinary sucrose excretion is presented as median excretion (mg). A 5 h urinary excretion of lactulose and rhamnose are presented as lactulose/rhamnose (L/R in g/g) ratios. Differences in urinary sucrose, L/R ratios and lactulose- and rhamnose recovery, respectively, between the lactoferrin- and placebo intervention were assessed using nonparametric statistics (Wilcoxon signed rank test, $P < 0.05$ was regarded as a statistical significant difference). Data are presented by Box-Whisker plots.

Results

None of the subjects suffered from gastrointestinal discomfort of any kind during the test days. No side effects of lactoferrin ingestion were observed.

The median urinary sucrose excretion was lower in the LF intervention (8.86; 3.61–25.4) compared to placebo (13.75; 2.89–24.62) but this difference did not reach statistical significance ($P = 0.31$; Figure 1).

The L/R ratio after ingestion of rhLF and indomethacin was significantly lower (0.028 (0.015–0.056); $P < 0.05$) than after the indomethacin challenge with placebo (0.036 (0.014–0.092)) (Figure 2). In Figure 2, the mean urinary lactulose/rhamnose ratio ± 2 standard deviations as the 95% reliability interval in healthy controls is depicted (0.014 \pm 0.016, $n = 31$). These values were obtained from control experiments in previous studies with healthy volunteers using the same protocol and analysis equipment by our group.

The observed difference in L/R ratio between the two interventions was because of a lower urinary lactulose recovery after lactoferrin administration compared to the placebo administration (0.52% (0.40–0.99) and 0.74% (0.26–0.92), respectively; $P = 0.054$). Urinary rhamnose recovery did not differ between the lactoferrin- and placebo administration (21.08% (9.12–28.56) and 21.00% (12.31–45.53), respectively; $P = 0.78$).

The urinary lactulose/rhamnose ratio after ingestion of 5 g rhLF (0.027; 0.02–0.03) did not differ from the urinary lactulose/rhamnose ratio after ingestion of water (0.03; 0.02–0.03).

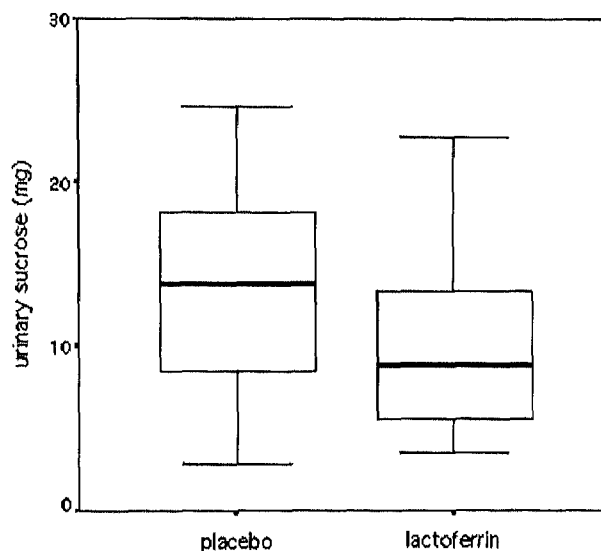


Figure 1 Median urinary sucrose excretion (mg) after two dosages of indomethacin (75 mg at 9 h and 50 mg indomethacin at 1 h, respectively before ingestion of a lactulose/rhamnose/sucrose solution) with or without ingestion of 5 g recombinant human lactoferrin at 24, 9 and 1 h before ingestion of the lactulose/rhamnose/sucrose solution ($P = 0.31$, $n = 15$, Wilcoxon signed rank test).

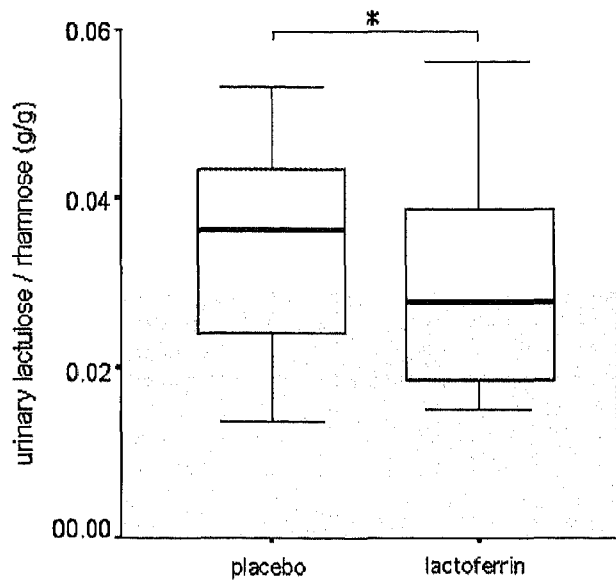


Figure 2 Median urinary lactulose/rhamnose (L/R) ratio after two dosages of indomethacin (75 mg at 9 h and 50 mg indomethacin at 1 h, respectively before ingestion of lactulose/rhamnose/sucrose solution) with or without ingestion of 5 g recombinant human lactoferrin at 24, 9 and 1 h before ingestion of the lactulose/rhamnose/sucrose solution (* $P < 0.05$, $n = 15$, Wilcoxon signed rank test). The gray area indicates the average ± 2 s.d. urinary lactulose/rhamnose ratios observed in control experiments in previous experiments ($n = 31$) as the 95% reliability interval for normal values in healthy controls.

In vitro lactoferrin-indomethacin binding study

After 7 min of incubation, no indomethacin was bound to LF: indomethacin recovery in the filtrate was 100%. After 22 min of incubation, 76% of the indomethacin appeared in the filtrate.

Discussion

It is well established that the administration of indomethacin in dosages comparable to that used in the present study increases small intestinal permeability about 2–3 fold (Bjarnason *et al*, 1986, 1989, 1993), which is in line with our results. Intestinal permeability after taking indomethacin for 7 days reverts back to normal within 1 week (Bjarnason *et al*, 1991). Hence, in the present study the washout period of 2 weeks was sufficient to exclude any carryover of the effects of indomethacin. Concomitant LF administration with indomethacin ingestion significantly attenuated the urinary lactulose/rhamnose ratio compared to placebo with indomethacin from 0.036 to 0.028, indicating that LF attenuated indomethacin-induced small intestinal permeability by a factor 1.4. Hence, LF administration does not completely prevent indomethacin-induced enteropathy. The observed lactulose/rhamnose ratio of 0.028, however, is within the normal range as measured previously

in control experiments in our lab, whereas 0.036 is above the normal range.

The difference in urinary lactulose/rhamnose ratios was caused by a lower urinary lactulose recovery after LF ingestion compared to placebo. Lactulose permeates the intestinal epithelium paracellularly and hence, urinary excretion after ingestion of the sugar drink provides an estimation of the paracellular permeation route. Rhamnose excretion is predominantly a parameter of transcellular permeation. Since we did not observe a difference in rhamnose excretion, the observed effect of LF on NSAID-induced hyperpermeability can be solely attributed to its protective effect on the paracellular permeation route. In the second *in vivo* study, we showed that LF did not affect small intestinal permeability in the unstressed small intestine. Hence, ingestion of LF does not inhibit normal small intestine permeability in humans, but provides an effective tool in the prevention of enteropathy in the small bowel.

Lactoferrin tended to exert a protective effect on gastro-duodenal permeability as measured by urinary sucrose excretion. The observed difference between LF and placebo did not reach statistical significance because of a wide range in results. It has been shown previously applying the urinary sucrose excretion method that aspirin administration increased gastroduodenal permeation (Lambert *et al*, 2001), indicating that NSAIDs inflict damage to gastric and duodenal mucosa. The observed trend in the present study warrants further investigation with sufficient power in order to elucidate the extent and pathophysiology of LF-associated protection of NSAID-induced damage to the stomach and the proximal duodenum.

Colostrum contains relatively large amounts of lactoferrin. A protective effect of a bovine colostrum preparation on indomethacin-induced increase in small intestinal permeability was previously observed. Colostrum supplementation during 7 days inhibited the indomethacin-induced hyperpermeability of the small intestine seen after 5 days of indomethacin intake compared to placebo. The placebo solution was an isoproteinaceous whey solution free of a number of growth factors that are present in the colostrum (Playford *et al*, 2001). No information was available about lactoferrin contents of the solutions used in this study. The results of the present study indicate that these effects could at least partly be attributed to LF present in the colostrum.

As will be explained below, the observed effect of LF in attenuating paracellular epithelial damage may be because of a protective effect on the regulation of the paracellular permeation route. LF may also interfere with another direct or indirect pathophysiological process by which indomethacin administration leads to gastroenteropathy such as the uncoupling of mitochondrial respiration, mediation of cytokine production, inhibition of cyclooxygenase-mediated prostaglandin production or induction of intracellular transcription factors.

It is well established that NSAID administration causes gastroenteropathy, thereby damaging the tight junctions

between enterocytes. This has a detrimental effect on the intestinal barrier function. NSAIDs may induce enteropathy by several distinctive mechanisms.

NSAIDs are able to uncouple oxidative phosphorylation, which results in reduction of intracellular ATP levels and, as a consequence, loss of control of the integrity of tight junctions. This is regarded as the first step in the development of gastrointestinal injury (Somasundaram *et al*, 1995, 1997). This uncoupling causes efflux of hydrogen and calcium ions from mitochondria and promotes oxygen radical damage (Bjarnason *et al*, 1993; Carafoli, 1987). Owing to this oxidative stress, tight junctions are damaged and paracellular permeability increases. LF is a well-recognized antioxidative agent because of its strong iron chelating capacity. Iron ions bound to LF are not able to participate in Fenton Chemistry in which the highly reactive hydroxyl radicals are produced. The chelation of iron by LF diminishes the production of hydroxyl radicals and consequently oxidative stress (Britigan *et al*, 1994). This may contribute to the observed protective effect of oral LF ingestion since indomethacin-induced enteropathy is in part caused by oxidative stress in the epithelium. The ferrochelating properties of LF also result in antimicrobial activity of LF in human plasma serum, as iron deprivation inhibits bacterial growth (Aguila *et al*, 2001). Additionally, LF exerts bactericidal activity against antibiotic-resistant bacteria *in vitro* independent of its iron-binding capacity (Bellamy *et al*, 1992; Nibbering *et al*, 2001). These findings indicate that LF may protect against indomethacin-induced enteropathy by preventing intrusion of bacteria in the epithelium of the small intestine, which occurs when the intestinal epithelium becomes more permeable and results in a further progression of the damage. Since the ferrochelating properties of LF may explain its protective effect on indomethacin-induced enteropathy, iron-free LF (apoLF) as used in the present study, may be more effective to protect against enteropathy than iron-saturated LF (holoLF). ApoLF has a maximal capacity to bind iron whereas holo has a limited capacity to bind free iron, although some iron binding can still occur.

The observed protective effects of oral LF ingestion may partly be because of the regulatory function of LF in the immune system. Oral lactoferrin ingestion decreases spontaneous and lipopolysaccharide-induced production of the proinflammatory cytokines IL-6 and TNF α by peripheral blood cells (Zimecki *et al*, 1998, 1999). Moreover, LF- and LF-derived peptides can stimulate the release of IL-8 from polymorphonuclear leukocytes, which activate neutrophils (Shinoda *et al*, 1996) and inhibit lymphocyte production of TNF α , IL-1 and IL-2 *in vitro* (Crouch *et al*, 1992). This mediating effect of LF on the local and systemic immune response may inhibit the proceeding of free radical-induced damage caused by local activation of the immune system because of NSAID-induced enteropathy. Regulation of cytokine production may be an important mechanism of action of LF resulting in protection of the integrity of the epithelial barrier, although data are equivocal. It was previously shown

that oral LF administration stimulates the production of the proinflammatory interleukin-18 (IL-18) in the mucosa of the small intestine in rodents with experimental metastasis (Kuhara *et al*, 2000). No data are available regarding the effects of oral LF ingestion on IL-18 production in any other physiological conditions.

It may be that the effect of LF on cytokine production is a receptor-mediated process. In that case, the origin of the rhLF administered in the present study may be essential. LF administration of animal origin may not exert the same effects as those observed in the present study since LF binding to its receptor is a species-specific process.

It is generally recognized that NSAIDs inhibit cyclooxygenase (COX) activity. This results in a decrease of prostaglandin (PG) synthesis and consequently a further deterioration of the epithelial barrier (Bjarnason *et al*, 1989; Hawkey and Rampton, 1985). It is unlikely that the protective effect of LF on the integrity of the epithelial wall involves PG production because existing knowledge does not provide any indications for a mediating effect of LF on PG production.

Hypothetically, binding of indomethacin to LF would prevent indomethacin-induced gastroenteropathy because indomethacin bound to LF may lack biological activity. In the *in vitro* LF/indomethacin binding study we showed that indomethacin did only bind LF in minute amounts at a slow rate. After 7 min of incubation, no indomethacin-LF binding was observed and after 22 min 76% of the indomethacin was recovered. This implies that at least 76% of the indomethacin was present as free indomethacin after 22 min. In previous studies, we showed that after 7 min almost half of the test drink ingested is emptied from the stomach, while after 22 min most if not all of the test drink is emptied from the stomach into the small intestine (Troost *et al*, 2001). Hence, in the present study, indomethacin entered the small intestine predominantly unbound to LF. The observed protective effect of oral LF administration can probably not be explained by LF-indomethacin binding. We previously showed that the major part of orally ingested LF survives gastric passage *in vivo* in adults, leaving most of the LF intact until it enters the intestine (Troost *et al* 2001).

The observed effects cannot be attributed to the presence of a protein load in the test drink. In contrast to LF, oral administration of casein leads to aggravation of NSAID-induced damage to the epithelial barrier, probably because transepithelial movement of casein induces enhanced mast cell activation and consequently mucosal injury (Miller *et al*, 1991).

Intestinal permeability is increased in numerous diseases. Approximately 50% of patients with active Crohn's disease have an increased small intestinal permeability (Bjarnason *et al*, 1983). Moreover, it has been shown that an increase in intestinal permeability precedes clinical manifestation of Crohn's disease (May *et al*, 1993). A compromised intestinal barrier function is regarded as an etiological factor in Crohn's disease (Meddings, 1997), celiac disease (Smecuol *et al*,

1997), viral gastroenteritis (Isolauri *et al*, 1989) and food allergy (Crowe & Perdue, 1992). This implies that controlling permeability is important in the control of these diseases. Ingestion of the two dosages of indomethacin as carried out in the present study increased small intestinal permeability approximately 2.5-fold, comparable to the increase in permeability observed in Crohn's disease (Wyatt *et al*, 1993). Hence, the protocol as used in this study may serve as an appropriate model for a variety of disease states in which small intestinal permeability is increased.

We conclude that orally administered rhLF exerts biological activity in the small intestine, resulting in inhibition of indomethacin-induced enteropathy. The exact mechanism of action of LF in attenuating enteropathy remains to be elucidated. It seems plausible that the antioxidative capacity of LF is involved in the attenuation of the indomethacin-induced stress. Binding of LF to its receptor, which may play a role in the pathophysiology of the observed effect of LF in the small bowel, is a species-specific process. Therefore, the results of the present study may be species-specific, depending on the origin of the LF. It seems less likely that LF interferes with the effects of indomethacin on PG synthesis or prevents the biological activity of indomethacin by binding it. Lactoferrin administration may be beneficial in the control of oxidative stress-induced damage of the small intestinal epithelium and especially in patients using NSAIDs by its attenuating effect on increased small bowel permeability.

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