Clin Investig (1993) 71:928–932

Clinical Pharmacology Original Article

The Clinical Investigator

Indomethacin decreases viscosity of gallbladder bile in patients with cholesterol gallstone disease*

C. von Ritter[1], A. Niemeyer[1], V. Lange[2], W. Möhrle[1], W.O. Richter[1], L. von Meyer[3], H. Brandl[1], R. del Pozo[1], D. Jüngst[1]

[1] Medizinische Klinik II, [2] Chirurgische Klinik, [3] Institut für Rechtsmedizin, Klinikum Großhadern, Ludwig-Maximilians-Universität München

Summary. There is experimental evidence that inhibition of cyclooxygenase with nonsteroidal anti-inflammatory drugs may decrease cholesterol gallstone formation and mitigate biliary pain in gallstone patients. The mechanisms by which NSAIDs exert these effect are unclear. In a prospective, controlled clinical trial we examined the effects of oral indomethacin on the composition of human gallbladder bile. The study included 28 patients with symptomatic cholesterol or mixed gallstones. Of these, 8 were treated with 3×25 mg indomethacin daily for 7 days prior to elective cholecystectomy while 20 received no treatment and served as controls. Bile and tissue samples from the gallbladder were obtained during cholecystectomy. Indomethacin tissue levels in the gallbladder mucosa, as assessed by HPLC, were 1.05 ± 0.4 ng/mg wet weight, a concentration known to inhibit effectively cyclooxygenase activity. Nevertheless, no differences between the treated and untreated groups were found in the concentrations of biliary mucus glycoprotein (0.94 ± 0.27 versus 0.93 ± 0.32 mg/ml) or total protein (5.8 ± 0.9 versus 6.4 ± 1.3 mg/ml), cholesterol saturation (1.3 ± 0.2 versus 1.5 ± 0.2), or nucleation time (2.0 ± 3.0 versus 1.5 ± 2.0 days). However, biliary viscosity, measured using a low-shear rotation viscosimeter, was significantly lower in patients receiving indomethacin treatment (2.9 ± 0.6 versus 5.6 ± 1.2 mPa.s; $P < 0.02$). In conclusion, in man oral indomethacin decreases bile viscosity without alteration of bile lithogenicity or biliary mucus glycoprotein content. Since mucus glycoproteins are major determinants of bile viscosity, an alteration in mucin macromolecular composition may conceivably cause the indomethacin-induced decrease in biliary viscosity and explain the beneficial effects of nonsteroidal anti-inflammatory drugs in gallstone disease.

Key words: Biliary mucus – Nonsteroidal anti-inflammatory drugs – Cholesterol nucleation time

In animal models of cholesterol gallstone disease nonsteroidal anti-inflammatory drugs (NSAIDs) decrease the tendency of bile to form gallstones [10]. The mechanisms responsible for this effect of NSAIDs are unclear. Inhibition of mucus secretion has been proposed as a potential mode of action. This view is based on the observation that in a number of animal models mucus hypersecretion is observed before crystal formation [11], and that in the prairie dog aspirin inhibits both cholesterol crystal formation and mucus secretion [10]. Recent studies on prairie dog gallbladder explants [15], however, failed to show an effect of aspirin on mucus secretion in concentrations which effectively inhibit prostaglandin synthesis. Furthermore, the relevance of the data collected in animal experiments for cholesterol gallstone disease in man is unclear. Studies in gallstone patients suggest that under aspirin treatment gallstone formation is reduced both during weight loss [2] and after successful litholytic therapy [7]. A recent prospective study by Rhodes et al [16] showed that in gallstone patients aspirin decreases mucus synthesis without a reduction in the mucus concentration of bile.

The present study was designed to define the effect of oral indomethacin on lithogenicity of bile in patients with cholesterol gallstone disease. To seek a better understanding of the mechanisms involved in the action of NSAIDs in gallstone disease a detailed analysis of bile was performed, including measurement of biliary lipids, proteins, mucus glycoproteins, and viscosity. Finally, to determine whether indomethacin treatment is sufficient to achieve effective drug levels in the tissue, indomethacin tissue concentrations were measured in the gallbladder mucosa. These measurements also served as index for patient compliance.

Abbreviation: NSAIDs = nonsteriodal anti-inflammatory drugs

* Dedicated to Prof. Dr. G. Paumgartner on the occasion of his 60th birthday

Material and methods

Experimental design and patients

The study was designed as a prospective, controlled clinical trial. Twenty-eight patients were studied. Exclusion criteria were acute cholecystitis with obstruction of the cystic duct, gastroduodenal ulcera, or hypersensitivity to indomethacin. Twenty patients received no treatment and served as controls. Eight gave informed consent to be treated with indomethacin 25 mg three times daily for 7 days prior to cholecystectomy and finished the full course of the 7-day treatment. Age, sex, number of gallstones and cholesterol content of gallstones of the patients in both groups are listed in Table 1.

Table 1. Characteristics of patients

	Untreated ($n=20$)	Indomethacin treated ($n=8$)
Mean age (years; range)	48 (23–71)	49 (29–69)
Sex (female/male)	15/5	8/0
Number of stones (solitary/multiple)	12/8	5/3
Cholesterol content of stone(s) (%)	58 ± 8	71 ± 9

Collection of samples

Bile was collected during cholecystectomy by complete aspiration of gallbladder bile with a 16-G needle. Aspiration was performed immediately after ligation of the cystic duct. Samples were kept under anaerobic conditions and processed within 30 min after aspiration. Aspirates contaminated with blood were not used. From the corpus of the gallbladder $2 \times 1 \times$ cm tissue samples were taken, frozen in liquid nitrogen and stored at -30°C.

Indomethacin tissue levels

To determine compliance and to control for interindividual differences in the absorption of indomethacin into the gallbladder mucosa, indomethacin tissue levels were measured in homogenized samples of the gallbladder mucosa using HPLC in a modified method as described by Shankar et al 1988 [18]. Briefly, samples of the gallbladder wall (2 cm^2) obtained during cholecystectomy were dissected sharply into the mucosal and muscular layer. The mucosa was homogenized using an Ultra Turrax T25 Homogenizer diluted with phosphate buffer and incubated for 2 h in a papain, cysteic acid, and EDTA solution to ensure complete release of indomethacin from the tissue matrix. The samples were centrifuged at 12000 rpm for 5 min. The supernatant was extracted using 2 ml diethyl ether and the organic layer evaporated to dryness in a 60°C waterbath. The dried extract was reconstituted in 30 µl acetonitrile/H_20(1:1) solution and a 20 µl aliquot of this solution was injected in the HPLC. A Beckmann (model 340 organizer) HPLC equipped with a Beckmann solvent delivery module (model 112) variable wavelength detector (model 165) and a Merck-Hitachi Chromato-integrator (model D 2500) was used. The analytical column was a Merck LiChrospher 60, RP-select B. Column temperature was 22°C and the flow rate 0.8 ml/min. For drug detection the column eluate was measured at 254 nm. Sensitivity and linearity were tested with standard solutions of indomethacin (0.1, 0.5, 1.0, 2.0, 5.0 ng/ml) disolved in methanol. Mefenamin (50 µl) was used as internal standard. Recovery was calculated by comparison of standard solutions added to drug-free tissue samples and standard solutions in methanol.

Analysis of bile

Nucleation time was determined as described by Holan [6]. For the analysis of biliary lipids samples were stored at -70°C. Cholesterol was determined colorimetrically with the Liebermann-Burchard reaction [1]. Phospholipids were measured as total biliary phosphate using the colorimetric assay of Fiske and Subbarow [4]. Total bile salts were determined by a modified 3α-hydroxysteroid dehydrogenase method [20]. The cholesterol saturation index of each sample was calculated according to Carey [3]. Total proteins were determined using a modified Lowry assay according to Jüngst et al. [8]. The pH values were determined immediately after aspiration in bile samples kept under anearobic conditions.

Mucus glycoproteins were separated from bile by gel permeation chromatography. Sepharose 2B-CL columns (80×2.5 cm) were loaded with 1 ml samples of bile and eluted with 0.2 M NaCl containing 0.03% sodium azide (NaN_3). Void volume was determined with blue dextran 2000. Mucin was quantitated in the void volume using the modified periodic acid–Schiff assay described by Mantle and Allen [13] with pig gastric mucin serving as standard.

Bile viscosity

Viscosity of gallbladder bile was determined as dynamic viscosity using a Low Shear Rotation Viscosimeter (Contraves, Zurich, Switzerland). The rotation viscosimeter allows accurate measurements of viscosity of both newtonian and nonnewtonian fluids. Dynamic viscosity was calculated from the shear forces exerted by a fluid placed in a rotating outer cylinder (2 T; 12 mm diameter × 11 mm height); shear forces were measured with an inner cylinder (2 T; 11 mm diameter). The applied shear rate in our experiments was in a range between 10 and 118/s. In all samples the viscosity curve reached a plateau at 60/s. Viscosity was calculated from triple measurements at this plateau and expressed as millipascals times second (mPa.s).

Statistical analysis

Values of each group of parametric data are presented as mean ± SEM. Non-parametric data (nucleation time) are expressed as median and range. Group comparison was performed for parametric data using unpaired Student's t test and for non-parametric data using the Mann-Whitney U test. The level of significance was set at $P<0.05$.

Results

Indomethacin tissue levels

A close linear detector response curve was found for indomethacin solutions in a range of 0.1–5 ng/ml. Recovery rates after extraction from drug-free mucosal homogenates were 94%. Duplicate analyses varied less than 5%. No indomethacin was measured in the gallbladder mucosa of untreated patients. In the treated group indomethacin tissue levels were 1.05 ± 0.4 ng/mg wet weight.

Analysis of bile

Despite these significant differences in indomethacin tissue concentrations no significant differences were found in pH, biliary lipids, or total protein content of bile (Table 2). Lithogenicity of bile, as determined by nucleation time, was also unaltered by indomethacin treatment. Furthermore, indomethacin failed to decrease biliary mucus glycoprotein content. However, as shown in Fig. 1 dynamic viscosity was significantly decreased in patients receiving indomethacin treatment.

Table 2. Effect of indomethacin on biliary lipids, total proteins, mucin glycoproteins, pH, and nucleation time

	Untreated ($n=20$)	Indomethacin ($n=8$)
Nucleation time median (range)	2 (1–4)	2 (1–14)
pH	7.5 ± 0.1	7.4 ± 0.1
Cholesterol (mmol/l)	21.5 ± 5.8	16.8 ± 5.6
Bile acids (mmol/l)	139.8 ± 20.0	107.9 ± 22.1
Phospholipids (mmol/l)	43.0 ± 5.8	38.4 ± 6.9
Total lipids (g/dl)	10.98 ± 1.46	9.5 ± 1.77
Cholesterol saturation index	1.29 ± 0.23	1.51 ± 0.18
Total proteins (mg/ml)	5.7 ± 0.9	5.9 ± 0.9
Mucus glycoproteins (μg/ml)	0.81 ± 0.22	0.95 ± 0.27

No significant alterations were observed in response to indomethacin

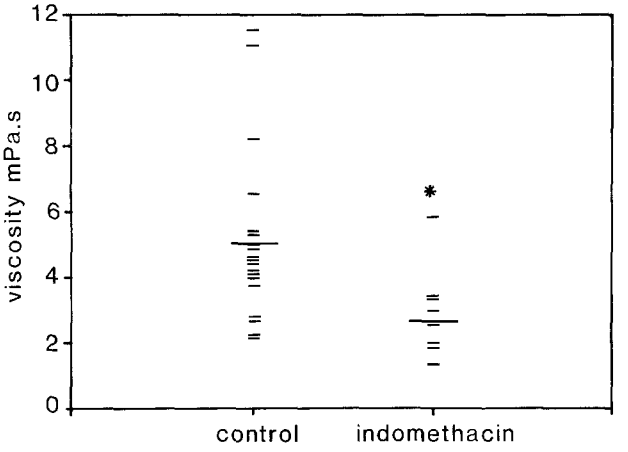

Fig. 1. Effect of indomethacin on bile viscosity. Indomethacin induced a significant decrease in dynamic viscosity ($P<0.05$)

Discussion

Indomethacin is a NSAID which inhibits prostaglandin synthesis. We measured indomethacin tissue levels in gallbladder mucosa homogenates in patients treated with 75 mg/day for 7 days. The tissue levels were 1.05 ± 0.4 ng/mg wet weight, a concentration known to inhibit effectively the enzymatic activity of prostaglandin synthetase [5,15]. The data collected in this study show that this inhibition of prostaglandin synthesis in pa-

tients with cholesterol gallstone disease leaves nucleation time, biliary lipids, cholesterol saturation, and proteins unchanged (Table 2). Biliary mucus hypersecretion precedes cholesterol crystal formation in the prairie dog [11]; in this animal high doses of aspirin prevent both mucus hypersecretion and cholesterol crystal formation [10]. It has therefore been speculated that aspirin may prevent gallstone formation by inhibition of mucus secretion. However, in our study, as observed previously in both prairie dogs [15] and man [16], indomethacin failed to decrease biliary mucus glycoprotein content. Therefore, other mechanisms appear to be responsible for the beneficial effects of cyclooxygenase inhibitors in gallstone disease.

A major finding of our study is the observation that indomethacin decreases the viscosity of gallbladder bile. Bile viscosity may play an important role in the formation of cholesterol crystals in human bile. Since bile flux through the cystic duct is inversely correlated to bile viscosity (Poiseuille's law), increases in bile viscosity may decrease the emptying of the gallbladder, thereby allowing cholesterol crystal growth. Recently indomethacin has been found to enhance postprandial emptying of the gallbladder in patients with cholesterol gallstone disease; indomethacin had no effect on gallbladder motility of healthy volunteers [14]. It is tempting to speculate that the indomethacin-induced decrease in bile viscosity observed in our study is the mechanism allowing improved gallbladder emptying. Further studies are needed to better define the correlation between gallbladder motility and bile viscosity.

Mucus glycoproteins are major determinants of bile viscosity. However, the data of the present study indicate that indomethacin decreases bile viscosity without changing biliary mucus glycoprotein content. One possibility to explain this apparent discrepancy is that indomethacin alters the oligomeric structure of the mucus molecule. The correlation between bile viscosity and alterations in the biliary mucus macromolecule has been carefully studied by Smith and coworkers [19]. Apparent viscosity and the size of the mucin polymer were found to be strongly dependent on pH, with a maximum of viscosity at pH 5.5. Our study showed no differences in pH in response to indomethacin. This makes it unlikely that differences in the degree of protonation account for the changes in viscosity observed. Studies of intestinal mucus synthesis [9] have shown that inhibition of cyclooxygenase with aspirin affects glycoprotein synthesis by at least two separate mechanisms: firstly, by reduced synthesis of peptides and, secondly, by reduced oligosaccharide assembly of the mucus glycoproteins. Aspirin appears to inhibit specifically acetylation of glucosamines [12]. Similar to these findings, aspirin-induced inhibition of mucus synthesis has also been observed in human gallbladder explants in two recent studies by Rhodes et al [16,17]. Taken together, there is experimental evidence showing that NSAIDs may interfere with mucin synthesis, possibly altering the degree of mucin glycosylation. Further studies are needed to clarify the effects of NSAIDs on the composition and structure of biliary mucins.

Acknowledgements. Part of the study was presented at the annual meeting of the American Gastroenterological Association, San Fransisco, 1992. The study was supported by grants from the Deutsche Forschungsgemeinschaft [Ri 584/1–1] and the Friedrich-Baur-Stiftung [74/91; 57/89]. The technical assistance of Benedikta Zündt and Carsten Meinecke is gratefully acknowledged.

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Received: May 3, 1993
Returned for revision: July 5, 1993
Accepted: July 9, 1993

Dr. C. von Ritter
Medizinische Klinik II
Klinikum Großhadern
Marchioninistraße 15
D-81366 München, Germany