Biochemical Pharmacology, Vol. 35, No. 23, pp. 4291–4295, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

EFFECT OF ACETYLSALICYLIC ACID ON GASTRIC MUCIN VISCOSITY, PERMEABILITY TO HYDROGEN ION, AND SUSCEPTIBILITY TO PEPSIN

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(*Received* 13 *March* 1986; *accepted* 9 *May* 1986)

Abstract—The effect of acetylsalicylic acid (aspirin) on peptic degradation of gastric mucin, its viscosity and the ability to retard the diffusion of hydrogen ion was investigated. The results of peptic degradation indicated that, in the absence of the drug, the rate of proteolysis was proportional to mucin concentration up to 400 μg and remained constant with time for up to 1 hr. Introduction of aspirin led to an enhancement in the rate of proteolysis. The apparent K_m value of pepsin toward mucus glycoprotein was 8.7×10^{-7} M in the absence of the drug and 6.9×10^{-7} M in its presence. Viscosity measurements showed a drop in mucin viscosity following preincubation with aspirin. This decrease was concentration dependent and at a 4.0×10^{-5} M concentration of the drug reached a value of 75%. Permeability studies revealed that preincubation with 2.0×10^{-5} M aspirin increased the permeability of mucin to hydrogen ion by 10%, while an 18% increase was obtained with 4.0×10^{-5} M aspirin. The results suggest that aspirin weakens the integrity of the gastric mucus layer by promoting its peptic degradation, decreasing viscosity, and reducing the ability to resist hydrogen ion penetration.

Aspirin when introduced intravenously or applied topically is known to cause acute gastritis and gastric erosions, and can also lead to chronic gastric ulcer [1–4]. Aside from changes in gross morphology, aspirin induces inhibition of ion transport in the gastric mucosal cells, causes a decrease in potential difference, and reduces the ATP content [5–7]. Furthermore, aspirin exerts a strong inhibitory effect on prostaglandin generation and oxidative phosphorylation, and thus affects the mucosal blood flow and the rate of gastric bicarbonate secretion [7–10]. Yet, the mechanism of gastric mucosal damage by aspirin is not well understood [11–12].

Histochemical and biochemical studies indicate that, at least partly, the noxious effects of aspirin are exerted through qualitative and quantitative changes in mucus glycoprotein, a component of gastric mucus which is responsible for the maintenance of the integrity of the protective mucus layer [13–15]. As the protective qualities of the gastric mucus layer depend strongly upon its viscous nature and the ability to resist the damaging effects of acid and pepsin, the alterations in mucus glycoprotein caused by aspirin may be reflected in the physicochemical characteristics of the mucus gel. Here, we present evidence that acetylsalicylic acid (aspirin) decreases the resistance of gastric mucus glycoprotein to peptic degradation and adversely affects the viscosity and the ability of the this glycoprotein to retard the acid diffusion.

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MATERIALS AND METHODS

Preparation of gastric mucin. Gastric mucus used for the isolation of mucus glycoprotein was obtained by instillation of six freshly dissected pig stomachs with buffered, pH 7.0, 2 M NaCl [16, 17]. Following dialysis and lyophilization, the mucus was dissolved in 6M urea and chromatographed on a Bio-Gel A-50 column, equilibrated in and eluted with 6 M urea–10 mM sodium phosphate buffer, pH 7.0. Fractions containing the excluded mucus glycoprotein peak were pooled, dialyzed against distilled water, and lyophilized. The removal of residual noncovalently bound protein from isolated glycoprotein was accomplished by equilibrium density gradient centrifugation in CsCl [18]. For this, the lyophilized glycoprotein was dissolved in 0.05 M phosphate buffer–0.15 M NaCl, pH 7.0, containing 42% (w/w) CsCl and centrifuged for 48 hr at 12° and 46,000 rpm in a Beckman 50Ti rotor. The mucus glycoprotein, recovered from the density gradient tubes with the aid of a Beckman fraction recovery system, was dialyzed exhaustively against distilled water and lyophilized.

Acetylsalicylic acid. The acetylsalicylic acid (aspirin; 2-acetoxybenzoic acid) powder, Lot No. 52F-0182, was obtained from the Sigma Chemical Co., St. Louis, MO. For the experiments, various concentrations of aspirin were prepared in 0.15 M NaCl.

Viscosity measurement. Viscosity determinations were performed with a Brookfield cone/plate digital viscometer, model LVTDCP equipped with a 1.565° cone and a constant (37°) temperature bath [19, 20]. Shear rates were varied from 1.15 to 230 sec^{-1}, and

the sample volumes were 0.5 ml. For the measurements, the glycoprotein dissolved at 30 mg/ml in 0.10 M NaCl–0.05 M phosphate buffer, pH 6.0, was mixed with various concentrations of aspirin, briefly sonicated, and left for 16 hr at 4° with gentle stirring. Prior to measurements, the samples were brought to 37°. To calculate the specific viscosity (η_{sp}), the measurements were also taken of buffer alone and buffer plus aspirin.

Hydrogen ion diffusion measurement. The diffusion of hydrogen ion through gastric mucus glycoprotein in the presence of aspirin was measured in a specially constructed Lucite chamber [21, 22]. In this device, the center panel separating the two compartments, filled on on side with 0.15 M HCl and on the other side with 0.15 M NaCl, contains a cylindrical sample port (1 mm thick and 7 mm diameter) of 38 μl capacity. The openings of the port to NaCl and HCl compartments are fitted with Millipore membrane (0.45 μm pore size) discs to hold the sample in place and at the same time to allow ready diffusion of hydrogen ion. The solutions in both sides of the port are agitated continuously by magnetic stirrers and the device is maintained at 37° in a thermostatically controlled Plexiglas chamber. The hydrogen ion concentration in the compartment containing NaCl is measured with a micro-pH electrode connected to an Orion digital ionanalyzer. The mucus glycoprotein, dissolved at 30 mg/ml in 0.15 M NaCl, was mixed with various concentrations of aspirin, briefly sonicated and incubated at 37° for 2 hr in a shaking water bath. An aliquot of sample was then transferred with a syringe into the diffusion port, and the change in pH in the compartment containing NaCl was recorded at 5-min intervals for up to 2 hr. The amount of hydrogen ion diffusing through the sample was calculated in moles/sec and the permeability coefficient in cm/sec [23]. In control experiments, the layer of mucus glycoprotein was substituted with 0.15 M NaCl and aspirin in 0.15 M NaCl. All experiments were repeated several times for reproducibility.

Peptic activity assay. The initial incubation mixtures for peptic activity assays at pH 1.8 consisted of the following components: mucus glycoprotein substrate, 100–600 μg; pepsin (porcine, 2700 units/mg protein), 0.16 to 1.28 μg; and aspirin, 0–6.0×10^{-5} M, in a final volume of 0.22 ml. In the standard assays 300 μg glycoprotein, 0 or 1.0×10^{-5} M aspirin, and 0.64 μg pepsin were used. The glycoprotein substrate and aspirin were prepared as concentrated stock solutions in diluted HCl, pH 1.8. The tubes containing the complete incubation mixtures were briefly sonicated and the reaction was initiated by the addition of pepsin [16]. Incubation was carried out at 37° for 30 min and the proteolysis was terminated by the addition of 1 ml of 0.1 M borate buffer, pH 9.0. The proteolytic activity of pepsin towards mucus glycoprotein was measured by following the release of α-amino residues by the trinitrophenylation method [24]. Tubes containing boiled pepsin and glycoprotein, and the tubes with pepsin but devoid of glycoprotein served as controls.

Analytical methods. The content and composition of carbohydrate in the purified mucus glycoprotein was determined by gas chromatography following methanolysis, re-N-acetylation and derivatization with silylating reagent [18]. The protein was measured by the method of Lowry *et al.* [25]. The content of associated lipids was determined following extraction of the glycoprotein with chloroform–methanol [19, 21]. The covalently bound fatty acids, released from the delipidated glycoprotein with methanolic KOH, were quantitated by gas–liquid chromatography [26]. All experiments were carried out in duplicate, and the results are expressed as means ± SD. Student's *t*-test was used to test significance, and P values of 0.05 or less were considered as significant.

RESULTS

The chemical composition of the undegraded mucus glycoprotein purified from pig gastric mucus is given in Table 1. The glycoprotein contained 14.3% protein, 64.4% carbohydrate, 20.1% associated lipids, and 0.4% covalently bound fatty acids. The carbohydrates were represented by fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid. The ratios of individual sugars were in the range of those found for other preparations of pig gastric mucus glycoprotein [16, 17]. The associated lipids were represented by neutral lipids (54.9%), glycolipds (33.8%) and phospholipids (11.3%), while the covalently bound fatty acids consisted mainly of hexadecanoic and octadecanoic acids.

Incubation of the purified mucus glycoprotein with pepsin resulted in the glycoprotein degradation as measured by the release of α-amino acid residues. The extent of peptic degradation of the glycoprotein increased with increasing amounts of pepsin up to 0.96 μg. The effect of mucus glycoprotein concentration on the proteolytic activity of pepsin is shown in Fig. 1. Under the assay conditions, the rate of proteolysis was proportional to a mucus glycoprotein concentration up to 400 μg and remained constant with time of incubation for up to 1 hr. The apparent K_m value for pig gastric mucus glycoprotein, based on the molecular weight of 2.0×10^6 [16], calculated from the double-reciprocal plots of the data was 8.7×10^{-7} M (Fig. 1).

Figure 2 illustrates the effect of aspirin concentration on the proteolytic activity of pepsin towards pig gastric mucus glycoprotein. The results

Table 1. Chemical composition of the purified pig gastric mucin

Component	Relative weight (%)
Fucose	9.7 ± 0.6
Galactose	21.6 ± 2.9
N-Acetylgalactosamine	13.1 ± 1.2
N-Acetylglucosamine	16.4 ± 1.5
Sialic acid	0.8 ± 0.1
Protein	14.3 ± 1.4
Associated lipids	20.1 ± 1.7
Covalently bound fatty acids	0.4 ± 0.1

Each value represents the means ± SD of triplicate analyses.

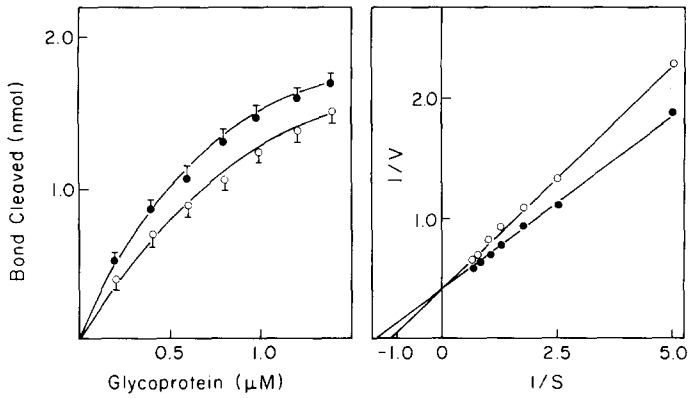

Fig. 1. Effect of gastric mucin concentration on the pepsin activity in the absence (○) and in the presence (●) of 1.0×10^{-5} M aspirin. The composition of the incubation mixtures was the same as described in the text, except that various concentrations (50–600 μg) of mucin were used. The data show the means ± SD of four separate experiments. In the double-reciprocal plots (right panel), $1/S = (M)^{-1}$ and $1/V =$ (nmoles·30 min^{-1}). The change in K_m value due to aspirin was significant at $P < 0.001$.

indicated that introduction of aspirin to the reaction mixtures led to an enhancement of the rate of glycoprotein digestion. The rate of enhancement was proportional to the aspirin concentration up to 3.0×10^{-5} M at which concentration a 35% increase in glycoprotein proteolysis was obtained. The apparent K_m value for peptic digestion of pig gastric mucus glycoprotein in the presence of aspirin was 6.9×10^{-7} M (Fig. 1). Results of experiments in which pepsin was preincubated for various periods of time (up to 1 hr) with aspirin prior to addition of mucus glycoprotein indicated no stimulatory effect on the activity of pepsin towards the glycoprotein substrate. However, when the glycoprotein was preincubated with aspirin prior to the addition of pepsin, a marked increase in peptic activity was observed.

Examination of the viscosity of glycoprotein at different pH indicated that the values did not vary significantly (less than 7%) over the studied (pH 2.0 to 7.0) range. Therefore, to avoid possible hydrolysis of carbohydrates from the glycoprotein, pH 6.0 was chosen in further studies. The effect of aspirin on the viscosity of pig gastric mucus glycoprotein is illustrated in Fig. 3. In the absence of aspirin, the specific viscosity of mucus glycoprotein ranged from 77 at the shear rate of $1.15 s^{-1}$ to 38 at the shear rate of 230 sec^{-1}. Preincubation with aspirin led to a drop in the glycoprotein viscosity. The observed decrease in mucus glycoprotein viscosity due to aspirin was concentration dependent and at its highest concentration (4.0×10^{-5} M) caused a 75% drop in viscosity.

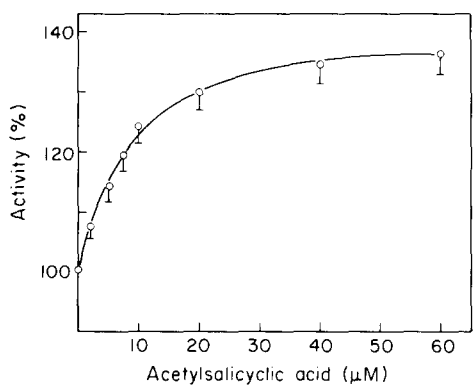

Fig. 2. Effect of aspirin concentration on the digestion of gastric mucin with pepsin. The assay conditions were the same as described in the text, except that various concentrations (0–6.0×10^{-5} M) of aspirin were used. The data show the means ± SD of four separate experiments. The rate of proteolysis enhancement by 3.0×10^{-5} M aspirin was significant at $P < 0.001$.

The effect of aspirin on the permeability of pig gastric mucus glycoprotein to hydrogen ion is shown in Fig. 4. The data illustrate that, following the initial period of equilibration, the permeability to hydrogen ion of mucus glycoprotein alone reached the value of 3.87 ± 0.34 moles/sec. Preincubation with aspirin led to a decrease of mucus glycoprotein ability to retard the diffusion of hydrogen ion. The permeability of mucus glycoprotein to hydrogen ion increased by 10% in the presence of 2.0×10^{-5} M aspirin, whereas 4.0×10^{-5} M aspirin increased the permeability of the glycoprotein by 18%. The data on permeability to hydrogen ion of pig gastric mucus glycoprotein in the presence of increasing concentrations of aspirin are summarized in Table 2.

DISCUSSION

The mucus component of the gastric mucosal barrier consists of a thick layer of a mucous gel overlying the gastric mucosa which confines the interaction between secreted bicarbonate and acid in such a way that a low pH is kept on the luminal side and a neutral pH is maintained on the mucosal side [1, 6, 27]. The thickness and physicochemical properties of the mucus layer remain in a delicate dynamic equilibrium controlled by the factors affecting the biosynthesis and secretion of mucus, and its breakdown through proteolysis or mucolytic agents [16, 28, 29]. While this delicate balance is maintained under normal

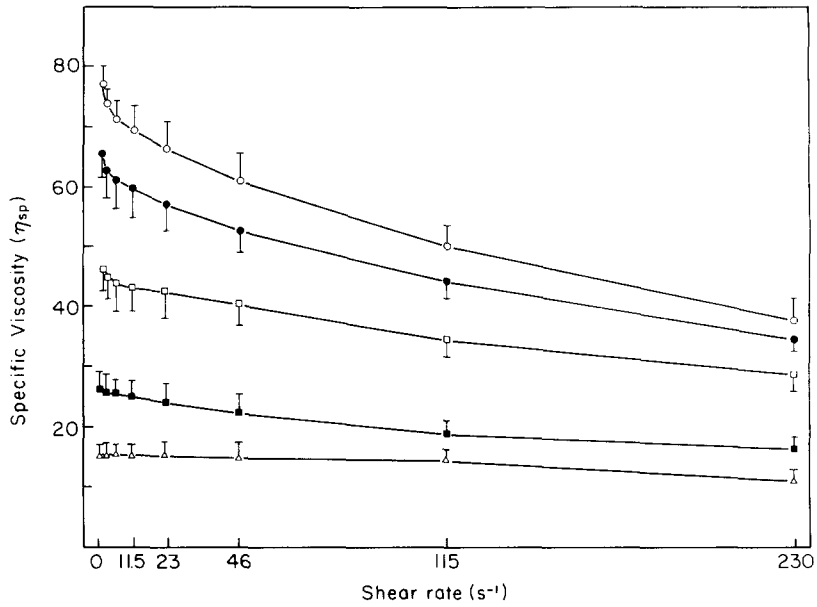

Fig. 3. Effect of aspirin on the viscosity of gastric mucin. Key: purified mucin (○), and mucin preincubated with aspirin at concentrations of 0.5×10^{-5} M (●), 1.0×10^{-5} M (□), 2.0×10^{-5} M (■), or 4.0×10^{-5} M (△). Values represent means ± SD of five separate experiments for each aspirin concentration. The changes in mucin viscosity due to aspirin at concentrations greater than 0.5×10^{-5} M were significant at $P < 0.001$.

physiological conditions, a number of noxious agents including aspirin and its metabolite, salicylic acid, when introduced into the stomach, readily disturb this state. The damage to gastric mucosa by aspirin can occur through its inhibitory action on the cyclooxygenase enzyme involved in prostaglandin synthesis and as a result of its conversion to salicylic acid which is a potent inhibitor of oxidative phosphorylation [7, 11, 30, 31]. In addition to these metabolic effects, aspirin is also known to be a strong mucolytic agent [28, 32], and thus could affect directly the physicochemical properties of the mucus layer. Indeed, the topical application of aspirin to gastric mucosa has been shown to weaken the gastric mucosal barrier by causing the release of the components of its mucus layer [28]. As the mucus layer constitutes the first line of mucosal defense, a number of functional properties of this layer could be affected as a consequence.

The results reported herein, obtained with pig gastric mucin which chemically resembles that of human [16–18], indicate that aspirin promotes the digestion of this glycoprotein by pepsin, decreases

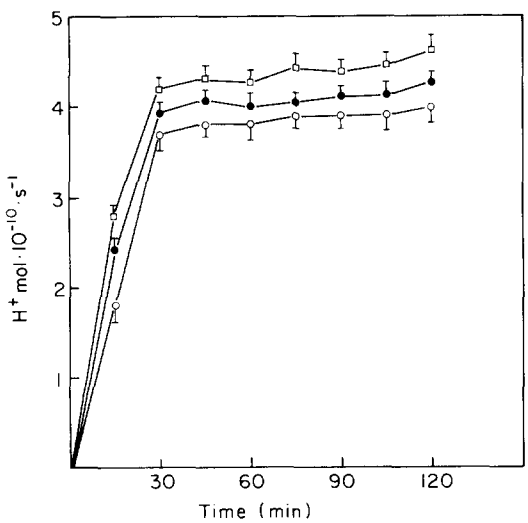

Fig. 4. Effect of aspirin on the permeability of gastric mucin to hydrogen ion. Key: purified mucin (○), and mucin preincubated with aspirin at concentrations of 2.0×10^{-5} M (●), or 4.0×10^{-5} M (□). Values represent means ± SD of five separate experiments for each aspirin concentration. The increase in mucin permeability with 2.0×10^{-5} M aspirin was significant at $P < 0.02$, whereas that with 4.0×10^{-5} M aspirin was significant at $P < 0.001$.

Table 2. Effect of aspirin concentration on the permeability of gastric mucin to hydrogen ion

Aspirin concn. (M)	Permeability coefficient ($cm \cdot 10^{-6} \cdot sec^{-1}$)
0	6.48 ± 0.53
0.5×10^{-5}	6.67 ± 0.57
1.0×10^{-5}	6.86 ± 0.59
2.0×10^{-5}	7.08 ± 0.64*
4.0×10^{-5}	7.65 ± 0.71†
Control (0.15 M NaCl)	68.60 ± 4.21

Each value represents the means ± SD of five separate experiments.

* $P < 0.02$ as compared with mucin alone.

† $P < 0.001$ as compared with mucin alone.

its viscosity, and reduces its ability to resist the hydrogen ion penetration. The data on peptic digestion showed that aspirin at doses which are known to cause gastric mucosal injury [28] produced up to 35% increase in mucin degradation. The evidence that aspirin causes an increase in the permeability of gastric mucin to hydrogen ion came from the comparison of the diffusion rate through glycoprotein with and without the drug. This permeability increased by 10% in the presence of 2.0×10^{-5} M aspirin and by 18% at 4.0×10^{-5} M. Aspirin also exerted a detrimental effect on the viscosity of gastric mucin. The effect was most pronounced at an aspirin concentration of 4.0×10^{-5} M where a 75% drop in mucin viscosity was observed.

The increase in degradation of gastric mucin due to aspirin may have far-reaching consequences on the integrity of the gastric mucosal barrier, since the destruction of the polymeric structure of mucin leads to the collapse of mucus gel matrix and thus compromises the first line of mucosal defense. While the mechanism by which aspirin enhances the activity of pepsin towards gastric mucin is not clear, the results of preincubation experiments suggest that this effect is due to the interaction of the drug with mucin rather than with pepsin. As to the detrimental effect of aspirin on the viscosity and permeability of gastric mucin, one can speculate that this may be due to the ability of the drug to interfere with the intermolecular and intramolecular hydrophobic forces which are so essential for the maintenance of the polymeric structure of this glycoprotein in the gastric mucus gel.

Acknowledgements—This work was supported by USPH Grant AM 21684-08 from the National Institute of Diabetes and Digestive and Kidney Diseases, NIH.

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