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HYDROGEN ION DIFFUSION IN DOG GASTRIC MUCUS GLYCOPROTEIN: EFFECT OF ASSOCIATED LIPIDS AND COVALENTLY BOUND FATTY ACIDS

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SUMMARY - The effect of neutral lipids, glycolipids and phospholipids associated with dog gastric mucus glycoprotein, and that of covalently bound fatty acids on the ability of glycoprotein to retard the diffusion of hydrogen ion was investigated. Purified mucus glycoprotein in its native form, placed between equimolar (0.155M) solutions of HCl and NaCl in a specially designed two-compartment chamber, caused a 90% reduction in permeability to hydrogen ion when compared with a layer of NaCl. Extraction of associated lipids lead to a 68% increase in permeability of the glycoprotein to hydrogen ion, while removal of the covalently bound fatty acids increased further the diffusion rate by 6%. Reassociation of the delipidated glycoprotein with its neutral lipids reduced the permeability to hydrogen ion by 34%, an 11% reduction was obtained with glycolipids, and 23% with phospholipids. Since neutral lipids account for 47% of the glycoprotein lipids, glycolipids 41.1% and phospholipids 11.9%, the quantitative decrease in permeability of the delipidated glycoprotein following its reassociation with phospholipids is 2.7 times greater than that of neutral lipids and 7.3 times greater than that of glycolipids.

INTRODUCTION - The viscous and slimy covering of the epithelial surfaces of gastric mucosa, referred to as mucus gel, is a heterogenous mixture of the molecules which find their way into the mucosal surface either by process of active secretion or by passive transudation (1,2). This highly hydrated layer of mucus is thought to protect the underlying mucosa from mechanical and enzymatic injury and confines the reaction between secreted HCO_3^- and H^+ entering the gel such a way that a low pH is kept on the luminal side and the neutral pH is maintained on the mucosal side (1-6). Another property of gastric mucus is its ability to retard the diffusion of hydrogen ion (7,8).

In previous reports we have shown that retardation of hydrogen ion by gastric mucus depends primarily on the polymeric structure of its major component, a high molecular weight glycoprotein called mucin, and the extent of this glycoprotein interaction with other mucus constituents such as serum

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albumin, IgA and lipids (9,10). Since lipids associated with mucus glycoproteins of gastrointestinal tract consist of neutral lipids, glycolipids and phospholipids (11,12), our aim in the present study was to elucidate the effect of individual classes of lipids on the retardation of H^+ diffusion by gastric mucin. The role of fatty acids covalently bound to mucus glycoprotein in the process of H^+ retardation was also determined.

MATERIALS AND METHODS - Gastric mucus used for the isolation of mucus glycoprotein was obtained by instillation of the freshly dissected dog stomach with buffered, pH 7.2, 2M NaCl (11). The instillates from nine stomachs were pooled, dialyzed against distilled water and lyophilized. Dried mucus was dissolved in 6M urea (at 10mg/ml) solution and chromatographed, in 5ml portions, on Bio-Gel A-50 column (2.0 x 150cm) equilibrated with 6M urea (11). Elution was achieved with 6M urea and the fractions containing the excluded mucus glycoprotein peak were pooled, dialyzed against distilled water and lyophilized. The removal of residual non-covalently bound protein from isolated mucus glycoprotein was accomplished by equilibrium density gradient centrifugation in CsCl (11). For this, the lyophilizate was suspended in 0.05M phosphate buffer - 0.15M NaCl, pH 6.9, containing 38% w/w CsCl and centrifuged for 48h at 12°C and 42,000rpm in a Beckman 50Ti rotor. The mucus glycoprotein were recovered from the density gradient tubes with the aid of a Beckman fraction recovery system, and were dialyzed exhaustively against distilled water and lyophilized.

Dried sample of purified mucus glycoprotein was delipidated by five consecutive extractions with chloroform-methanol (2:1, v/v), each time for 24h. The extracts were filtered through Millipore FH (0.5μm) filters to retain the insoluble glycoprotein residue, and the lipids contained in the filtrates were dried (11). The dry lipid residues were dissolved in a small volume of chloroform and separated on silicic acid (11-200 mesh) column (0.9 x 35cm) into three major lipid classes: neutral lipids, glycolipids and phospholipids (11). The delipidated glycoprotein was dialyzed against distilled water and lyophilized.

Reassociation of the delipidated glycoprotein with its total lipids and with the individual classes of lipids was carried out in 0.1M NaCl - 0.05M phosphate buffer, pH 7.2, containing 0.02% NaN_3 (10). The delipidated glycoprotein dissolved in the above buffer was added to the suspension of lipids in the same buffer and the mixture was sonicated for 5 min after which it was transferred to a shaking water bath and incubated at 37°C. After 2h, the mixture was dialyzed against distilled water and lyophilized.

The diffusion of hydrogen ion through mucus and its glycoprotein preparations was measured in a specially constructed Lucite chamber, design of which is illustrated in Fig. 1. In this device, the center panel separating the two compartments filled on one side with 0.155M HCl (pH 0.9) and on the other side with 0.155M NaCl (pH 6.5), contains a cylindrical port (1mm thick and 7mm in diameter) of 38μl capacity. The openings of the port to NaCl and HCl compartments were covered with a Millipore membrane (0.45μm pore size) discs to hold the investigated sample in place and at the same time to allow ready diffusion of hydrogen ion. The solutions on both sides of the port were continuously agitated by magnetic stirrers and the device was maintained at 37°C in a thermostatically controlled plexi-glass chamber. Hydrogen ion concentration in the compartment containing NaCl was measured with a micro-pH electrode connected to Orion digital ionanalyzer.

The mucus and the isolated glycoprotein in its native and modified forms were dissolved in 0.155M NaCl (at 30mg/ml), briefly sonicated and then incubated at 37°C for 2h in a shaking water bath. An aliquot of sample was transferred with a syringe through the top opening into the diffusion port

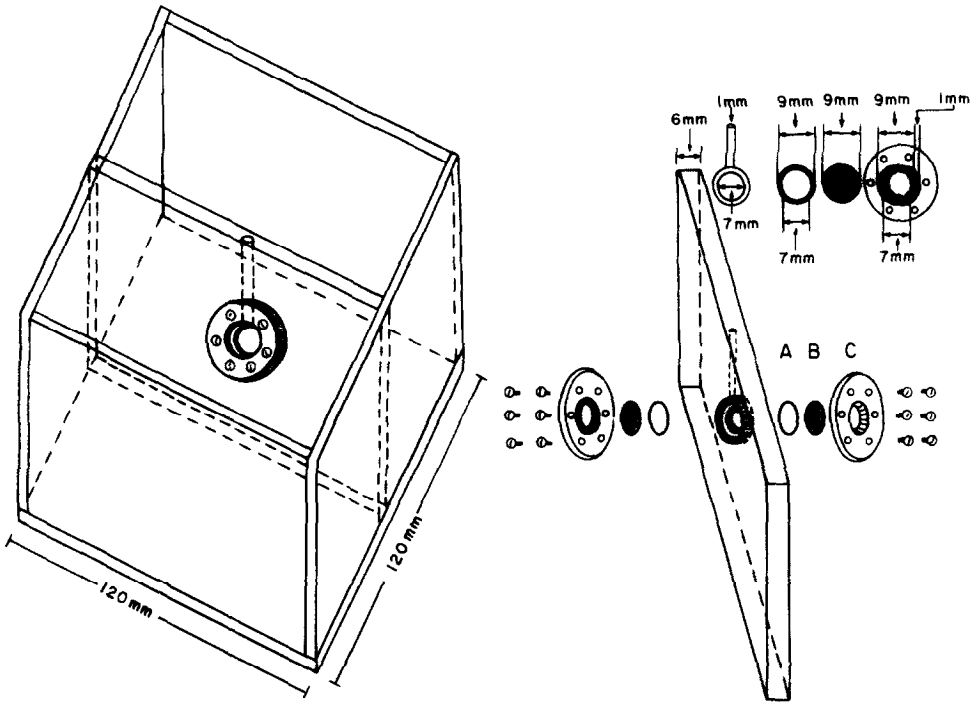

Fig. 1. Schematic illustration of the Lucite chamber used to measure the hydrogen ion diffusion. The device consists of two identical (6.0 x 12.0 x 7.0cm) compartments separated by a center panel containing the sample port. The hydrogen ion concentration was measured with a micro-pH electrode in the compartment containing NaCl solution.

and the time required for the pH in the compartment containing NaCl to change by one pH unit was recorded at 5 min intervals. The amount of hydrogen ion diffusing through the barrier was calculated in mol/second and permeability coefficient in cm/sec (13). In control experiments, the layer of mucus was substituted with 0.155M NaCl. All experiments were repeated six to ten times for reproducibility. Statistical analysis was performed using Student's t-test.

The protein content of samples was measured by the method of Lowry et al (14) with bovine serum albumin as standard. The content and composition of carbohydrates was determined by gas-liquid chromatography following methanolysis, re-N-acetylation, and derivatization with silylating reagent (15). The content of total lipids was determined gravimetrically, neutral lipids were quantitated according to procedures in (16), phospholipids by the method of Lowry and Tinsley (17), and glycolipids by analysis of their carbohydrate component (16). Removal of the covalently bound fatty acids from the delipidated mucus glycoprotein was accomplished with hydroxylamine (18).

RESULTS - The chemical characteristics of dog gastric mucus and its mucus glycoprotein used in the hydrogen ion diffusion experiments are given in Table I. The purified mucus glycoprotein, in comparison to native mucus, contained four times more carbohydrate and two times more lipid/g of dry sample. Among the major lipid classes in mucus, the neutral lipids accounted for 55% of the total lipids, glycolipids 34.6%, and phospholipids 10.5%. Of

Table I. Composition of dog gastric mucus and its purified mucus glycoprotein

Constituent	mg/100mg	
	Mucus	Glycoprotein
Protein	67.5 ± 15.3	18.9 ± 2.1
Carbohydrate	12.7 ± 1.9	52.3 ± 6.1
Total lipids	18.9 ± 2.1	29.4 ± 1.6
Neutral lipids	10.0 ± 1.6	12.7 ± 1.4
Glycolipids	6.3 ± 0.8	11.1 ± 1.3
Phospholipids	1.9 ± 0.2	3.2 ± 0.4
Covalently bound fatty acids	N.D.	0.3 - 0.4 ± 0.1

Each value represents the means ± SD of triplicate analyses. N.D. = not determined.

the total lipids isolated from the purified glycoprotein, 47% were represented by neutral lipids, 41.1% by glycolipids and 11.9 by phospholipids.

The neutral lipids derived form both types of samples exhibited similar composition, and were comprised of free fatty acids, cholesteorl, cholesteryl esters, and mono-, di- and triglycerides. In native mucus, the free fatty acids accounted for 48.5% of the neutral lipids, cholesterol 21.2%, cholesteryl ester 10.8%, triglycerides 16.5%, and mono- and diglycerides 3.1%. The neutral lipids derived from the purified mucus glycoprotein contained 41.1% free fatty acids, 32.3% cholesterol, 12.2% cholesteryl esters, 10.2% triglycerides, and 1.2% mono- and diglycerides.

The phospholipids constituted 1.9% of the dry weight of native mucus, and their content increased to 3.2% in the purified mucus glycoprotein. The major phospholipids identified in both samples were phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin and lysophosphatidylcholine. These five compounds accounted for 81.6% of the total phospholipids in mucus, and 87.6% in the mucus glycoprotein. The glycolipids of mucus and the purified mucus glycoprotein were composed mainly of glyceroglucolipids, but each sample also contained simple glycosphingolipids. The latter compounds constituted about 6.8% of the total glycolipids of mucus and 0.5 - 0.7% of the glycolipids of mucus glycoprotein, and were comprised mainly of glucosyl- and lactosylceramide. The glyceroglucolipids of

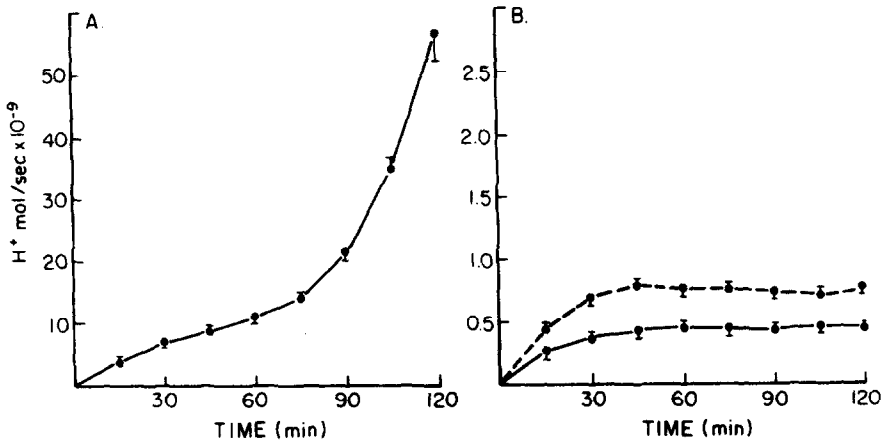

Fig. 2. Rate of change of hydrogen ion concentration in the NaCl compartment, as a result of its diffusion through the unstirred layer of 0.155M NaCl (A), and gastric mucus glycoprotein (B) in its native (●—●—●) and delipidated (●--●--●) forms. Values represent means ± SE of six separate experiments for each sample.

both types of samples were comprised of neutral (60%) and sulfated (40%) compounds.

The covalently bound fatty acids, released from the delipidated glycoprotein with hydroxylamine, constituted 0.3 - 0.4% of the dry weight of mucus glycoprotein, and consisted mainly of hexadecanoic and octadecanoic acids.

Figure 2 illustrates the rate of change in hydrogen ion concentration in the NaCl compartment, as a result of its diffusion through the sample port containing the unstirred layer of 0.155M NaCl (control) or mucus glycoprotein preparation. With only NaCl solution in the sample port (Fig. 2A), the permeability of this layer to hydrogen ion increased moderately with time for the first 60 min, raising rapidly thereafter the reached a steady state after 4h when the pH in the NaCl compartment fell below 3.0. Substitution of NaCl layer with the purified mucus glycoprotein had a profound deterimental effect on the rate of hydrogen ion diffsuion (Fig. 2B). The initial raise in hydrogen ion permeability occurred much (about 10 times) slower and a steady state was achieved at 40 min into the experiment when the pH in the NaCl compartment remained around 6.0. Significant increase ($p > 0.01$) in permeability of mucus glycoprotein to hydrogen ion was obtained following

Table II. Permeability to hydrogen ion of the purified dog gastric mucus glycoprotein in its native and modified forms

Type of material	No. of Expt.	Permeability (mol/sec) x 10^{-10}	Permeability coefficient (cm/sec) x 10^{-6}
Native mucus	7	4.56 ± 0.40	7.64 ± 0.82
Purified mucus glycoprotein	10	4.17 ± 0.31	6.98 ± 0.64
Delipidated glycoprotein	10	7.03 ± 0.52	11.78 ± 1.01
Delipidated glycoprotein following removal of covalently bound fatty acids	7	7.47 ± 0.39	12.52 ± 0.80
Lipid reassociated glycoprotein	9	4.38 ± 0.28	7.34 ± 0.58
Delipidated glycoprotein reassociated with neutral lipids	6	4.62 ± 0.43	7.74 ± 0.89
Delipidated glycoprotein reassociated with glycolipids	6	6.23 ± 0.35	10.44 ± 0.72
Delipidated glycoprotein reassociated with phospholipids	6	5.43 ± 0.24	9.10 ± 0.49
Control (0.155M NaCl)	8	40.92 ± 1.50	68.60 ± 3.09

Values represent means ± SE.

its delipidation. However, even with such modified glycoprotein a steady state was also achieved after 40 min (Fig. 2B).

The data on permeability to hydrogen ion of the purified dog gastric mucus glycoprotein in its native and modified forms are presented in Table II. The results show that permeability of purified mucus glycoprotein to hydrogen ion was slightly less than that of the native mucus. Extraction of associated lipids from the glycoprotein lead to a substantial (68%) loss in its ability to retard the hydrogen ion diffusion. This effect, however, was reversible and nearly complete restoration of the retardation of hydrogen ion diffusion was achieved following reassociation of the glycoprotein with its lipids. Further loss (about 6%) by the delipidated glycoprotein of the ability to retard the diffusion of hydrogen ion occurred following removal of the covalently bound fatty acids.

To determine the extent to which each of the three major classes of lipids associated with gastric mucus glycoprotein contribute to the retardation of hydrogen ion diffusion, permeability measurements were performed on the delipidated glycoprotein following its reassociation with neutral lipids, glycolipids, and phospholipids. The data indicate that following reassociation of the delipidated glycoprotein with its neutral lipids, permeability of the glycoprotein decreased by 34%. Reassociation of the delip-

idated glycoprotein with its phospholipids reduced the permeability to hydrogen ion by 23%, while the permeability of the delipidated glycoprotein following reassociation with its glycolipids decreased by 11%. The above results, thus indicate that all three classes of lipids associated with gastric mucus glycoprotein participate in the retardation of hydrogen ion diffusion, but their effect is not equal.

DISCUSSION - Among the mechanisms by which gastric mucus protects the underlying mucosal surface against the damaging effects of the high acid concentration of luminal contents is the retardation of hydrogen ion diffusion (7-10, 19-21). These properties of mucus are directly related to its ability to immobilize water molecules, a characteristic which is dependent on the gel-like nature of mucus. Because of its multiconstituent nature, elucidation of the role of mucus in the retardation of hydrogen ion diffusion requires an understanding the way in which each component contributes to this protective function. Utilizing a sintered glass barrier technique, we have shown recently that the ability of gastric mucus to retard the diffusion of hydrogen ion depends mainly on the integrity of its mucus glycoprotein and the extent of this glycoprotein interaction with proteins and lipids (9,10).

Since gastric mucus glycoprotein in its purified form contains about 29% of non-covalently bound lipids and also has 0.3 - 0.4% of covalently linked fatty acids (11,18), the experiments described in this report were designed to determine the contribution of these lipids to the retardation of hydrogen ion diffusion. The results obtained indicate that extraction of associated lipids was accompanied by a 68% increase in permeability of the delipidated glycoprotein to hydrogen ion, while removal of the covalently bound fatty acids increased further the rate of hydrogen ion diffusion by 6%. Thus, the associated lipids but not the covalently bound fatty acids appear to play a major role in the retardation of hydrogen ion diffusion by mucus glycoprotein. This effect may be the result of hydrophobicity imparted to mucus glycoprotein by the associated lipids. It should be noted that

the resistance of gastric mucus to aqueous solubilization under physiological conditions is well known phenomenon (1,3).

Evidence that contribution to the retardation of hydrogen ion diffusion of various lipids associated with mucus glycoprotein is different comes from the data on permeability of the delipidated glycoprotein following reassociation with each of its three major lipid classes. By reacting the delipidated glycoprotein with its neutral lipids a 34% decrease in the permeability of glycoprotein to hydrogen ion was obtained. At the same time the reassociation of the delipidated glycoprotein with its glycolipids, content of which is similar to that of neutral lipids, decreased the permeability of glycoprotein to hydrogen ion only by 11%. The greatest quantitative effect was obtained, however, with phospholipids. Since phospholipids account for 11.9% of the glycoprotein lipids, the quantitative decrease in permeability of the delipidated glycoprotein as a result of its interaction with phospholipids is about 2.7 times greater than that of neutral lipids and 7.3 times greater than that of glycolipids.

The way by which phospholipids exert their effect on the retardation of hydrogen ion diffusion by mucus glycoprotein is not readily apparent. However, in the light of our recent studies on topography of lipids within the mucus glycoprotein molecule which showed that interaction of phospholipids with glycoprotein involves its nonglycosylated domain (11,12), it is possible that this retardation effect of phospholipids may be due to their ability to stabilize the extended macromolecular structure of mucus glycoprotein. The hydrophobic nature of these nonglycosylated domaines of mucus glycoprotein molecules in the stomach, would thus create an environment impeding the hydrogen ion penetration. That mucosal surface of the alimentary tract, in parts exposed to acid, is highly hydrophobic has been recently demonstrated (22). Taken together, these data strongly implicate lipids in the gastric mucosal defense mechanism.

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