Biomaterials 54 (2015) 1–8

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Food-associated stimuli enhance barrier properties of gastrointestinal mucus

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ARTICLE INFO

Article history:
Received 12 November 2014
Received in revised form
18 February 2015
Accepted 27 February 2015
Available online 27 March 2015

Keywords:
Oral drug delivery
Mucus
Mucosal barriers
Food effect
Diffusion

ABSTRACT

Orally delivered drugs and nutrients must diffuse through mucus to enter the circulatory system, but the barrier properties of mucus and their modulation by physiological factors are generally poorly characterized. The main objective of this study was to examine the impact of physicochemical changes occurring upon food ingestion on gastrointestinal (GI) mucus barrier properties. Lipids representative of postprandial intestinal contents enhanced mucus barriers, as indicated by a 10–142-fold reduction in the transport rate of 200 nm microspheres through mucus, depending on surface chemistry. Physiologically relevant increases in [Ca^{2+}] resulted in a 2–4-fold reduction of transport rates, likely due to enhanced cross-linking of the mucus gel network. Reduction of pH from 6.5 to 3.5 also affected mucus viscoelasticity, reducing particle transport rates approximately 5–10-fold. Macroscopic visual observation and micro-scale lectin staining revealed mucus gel structural changes, including clumping into regions into which particles did not penetrate. Histological examination indicated food ingestion can prevent microsphere contact with and endocytosis by intestinal epithelium. Taken together, these results demonstrate that GI mucus barriers are significantly altered by stimuli associated with eating and potentially dosing of lipid-based delivery systems; these stimuli represent broadly relevant variables to consider upon designing oral therapies.

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1. Introduction

Mucus consists of a highly branched protein network, the major structural component of which is mucin glycoproteins [1]. Mucus also contains approximately 2% lipids and 90–95% salts, cells, electrolytes, other cellular debris, and water [2,3]. Intestinal mucus functions as a robust barrier to control the passage of harmful molecules and organisms such as bacteria, but allows efficient transport of nutrients across the epithelium. These conflicting functions are significant to establishing effective drug and nutrient transport across intestinal mucosa [4].

Ingested lipids are known to impact intestinal absorption. Most nutrients are lipid soluble; vitamins and cholesterol are solubilized in mixed micelles, which are formed from food-associated levels of bile salts and phospholipids [5]. The influence of lipids on orally delivered drug compound absorption across intestinal mucosa originates in part from colloidal structures the lipids form [6]. Lipids are also endogenously present in mucus, and include cholesterol, ceramide, palmitic acid, stearic acid, oleic acid, linoleic acid, and other free fatty acids [7,8]. It has been demonstrated that removal of lipids from airway mucus significantly alters viscoelastic properties [9,10]. Extraction of lipids from gastric mucus led to 80–85% decrease in viscosity [11]. Lipid content and composition are also known to affect the viscoelasticity in expectorated airway secretions from cystic fibrosis patients [12,13]. Furthermore, enhancement of mucociliary transport capacity has been correlated with the recovery of phospholipid content in airway mucus [14]. However, in spite of the tremendous physiological relevance of exposure of intestinal mucus to lipid mixtures originating from food or drug

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http://dx.doi.org/10.1016/j.biomaterials.2015.02.118
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delivery systems, the potential impact of exogenous lipids on barrier properties of intestinal mucus has not been explored.

Mineral salts are also associated with food intake, and comprise a mass percentage up to 1% in mucus [2]. Mucus properties depend in part on ionic interactions between mucins, and salts can thus directly alter mucus structure [15–17]. Monomers of the main secreted intestinal mucin, MUC2, form intermolecular links with [Ca^{2+}] that increase mucus viscoelasticiy [17,18]. After food arrival, [Ca^{2+}] concentration varies in the small intestine. Reported calcium ion concentration prior to eating is between 4 and 5 mM, and it could reach up to 20 mM after eating [19]. While multiple reports have demonstrated the impact of [Ca^{2+}] on physical properties of mucin solutions [16,17], the impact of exogenous [Ca^{2+}] on transport through native intestinal mucus has not been characterized.

Gastrointestinal pH also plays an important role in modulating drug delivery and nutrient absorption [20]. After food arrival, median small intestinal pH is approximately 5.4, whereas median fasting pH is close to 6.1. However, pH values fluctuate between individuals from a minimum as low as pH 3.1 to a maximum of pH 6.7 [21]. Acidity is known to alter gastric mucin solution rheological and structural properties [22]. Decreasing pH has been demonstrated to induce gastric mucin aggregation and increase gastric mucin solution viscosity [23,24]. It has been suggested that this is due to neutralization of glycosidic residues, however analysis of native and deglycosylated gastric mucin aggregation at low pH using atomic force microscopy indicated that oligosaccharide side chains are not required for mucin aggregation to occur [25]. It is unclear if similar pH effects are also observed in intestinal mucus, especially as the main gel-forming mucin in intestine (MUC2) is different from that in stomach (MUC5AC).

In this investigation, the impact of exposure to lipids and physicochemical stimuli, including pH and [Ca^{2+}] variations, associated with eating on particle transport through intestinal mucus has been investigated using microstructural analysis and real-time multiple particle tracking (MPT). MPT is a powerful technique that enables probing of particle–environment interactions by tracking the motion of hundreds of individual microspheres [26,27]. Diffusive particle transport is relevant as it probes the barrier properties of mucus microenvironment, in particular to micro-scale entities such as drug delivery systems or microbes. Particle solutions containing lipids, varied pH levels, and varied [Ca^{2+}] were directly exposed to native mucus collected from porcine small intestine, or intact excised small intestine tissue from mouse. Impact of exposure to stimuli *in vivo* was tested by oral dosing of lipids to rats.

In summary, while it has been indicated that endogenous lipids impact mucus viscoelastic properties, and [Ca^{2+}] and pH changes are significant to mucin solution viscoelastic properties, the majority of relevant studies have been conducted on mucins isolated from non-intestinal (e.g., airways, stomach) anatomical sites. This is particularly important given the variations in mucins expressed at different anatomical locations [28]. The impact of exogenous lipids and other physicochemical intestinal lumen changes associated with food intake on transport properties of intestinal mucus have not been characterized. Here, we utilize MPT and structural analysis to demonstrate that food-associated physicochemical stimuli significantly alter barrier properties of intestinal mucus, with important implications pertaining to physiological control of exposure to ingested microparticles and microbes, and oral drug delivery.

2. Materials and methods

2.1. Preparation and characterization of microspheres and test media

Fluorescently labeled yellow-green FluoSpheres (Invitrogen Molecular Probes, Carlsbad, CA) were used to prepare particle suspensions. Amine-, carboxylate-, and sulfate-modified microspheres (200 nm diameter, 2% solids in distilled water with 2 mM azide) were diluted in various test media (Table 1) [29] for a final particle concentration of 0.0025 wt.-%. The different particle surface functionalities were utilized to test the impact of varied chemistries on interaction with mucus components during transport through mucus. Fed intestinal state was mimicked with maleate buffer (pH 6.5, 10 mM $CaCl_2$), 12 mM bile salt (sodium taurodeoxycholate, NaTDC), 4 mM phospholipids (lecithin), and a lipid mixture comprised of 35 mM soybean oil, 30 mM sodium oleate, and 15 mM monoglycerol. The solution was mixed on a stirring plate at 37 °C with a continuous magnetic stirring at 300 rpm. Physiologically relevant pH values of 5.5 and 6.5 were obtained by adjusting NaOH concentration in maleate buffer (10 mM in $CaCl_2$) to 30 mM and 40 mM. A low pH of 3.5, indicating extreme conditions in the upper intestine where stomach contents are released, was obtained by not adding NaOH into maleate buffer. [Ca^{2+}] concentrations of 5, 10, and 20 mM at pH 6.5 were achieved by changing $CaCl_2$ concentration in maleate buffer. The particle sizes and zeta potentials (ζ-potentials) of the polystyrene microspheres were determined using dynamic light scattering (SI text).

2.2. Native mucus collection and preparation

Porcine intestines (Research 87, Boylston, MA) were obtained from a local abattoir within 2 h of slaughter. Native mucus was scraped with a spatula from pig jejunum and stored at −80 °C until use, as previously described [30].

2.3. Ex vivo preparation of excised mouse intestine

Intestinal tissue segments approximately 1 cm in length were taken approximately 10 cm from the exit of the stomach, corresponding to the jejunum, from 3 week old FVB/N type mice. Mice were euthanized via CO_2. Intestine fragments were cut open to expose the intestinal lumen, and placed into a chamber on a microscope slide maintained in a humidified environment.

2.4. In vivo dosing of lipid

Male rats (Sprague–Dawley), 70–90 days old, 300–375 g in weight, were dosed 2 ml of control (water) or test lipid system (soybean oil) by oral gavage. Prior to dosing, the soybean oil was stained with 0.01% Sudan IV solution, allowing for observation of soybean oil solution in the gastrointestinal tract using fluorescence microscopy after oral dosing. Rats were anesthetized using 2.5–3% isoflurane vapor according to IACUC approved protocols. The abdominal cavity was entered by way of a 2 cm midline abdominal skin incision positioned 1 cm below the diaphragm along the linea alba. Using a 22 gauge needle, 50 μl carboxylate-modified microspheres 200 nm in diameter diluted in PBS (0.0025 wt.-%) were injected into the duodenal lumen. Rats were kept anesthetized under 2.5% isoflurane on a warm blanket for 30 min to allow microspheres to disperse throughout the lumen. Intestinal (jejunum) tissue segments approximately 1 cm in length were excised approximately 10 cm from the exit of the stomach. Microspheres were imaged using fluoresence microscopy of intact tissue for tracking experiments. Approximately 1 cm segments of duodenum were collected for histology.

2.5. MPT of microspheres in intestinal mucus

The trajectories of fluorescently labeled microspheres were captured and recorded with a frame rate of 30 fps for 20 s using a 12.5 megapixel cooled Olympus DP70 digital color camera (Olympus, Center Valley, PA) mounted on an inverted Olympus IX51 microscope attached with X-Cite 120 fluorescence system (EXFO, Mississauga, Ontario, Canada). Excised tissue obtained after *in vivo* exposure to stimuli or for *ex vivo* exposure was placed within chambers formed by 0.8 mm deep silicone gaskets (Grace Bio-Labs) attached to microscope slides. Scraped mucus was placed within non-fluorescent 8-well polystyrene medium chambers (Thermo Fisher Scientific, Rochester, NY). Diluted particle suspension (10 μl, 0.0025% wt/vol) was deposited with minimal perturbation onto approximately 200 μl of scraped native mucus or 10 mm excised mouse explant tissue segments. The mucosal specimens were covered and equilibrated for 2 h at 25 °C in a humid chamber prior to microscopy. Trajectories of at least 100 microspheres were analyzed for each experiment and three experiments were performed from three different mucus specimens for each experimental setup to account for mucus variability. Trajectories

Table 1
Bio-relevant media dosed to mucosal surfaces.

Maleate buffer	Triz-Ma	100	mM
	NaCl	65	mM
	$CaCl_2$	5–20	mM
	NaN_3	3	mM
	NaOH	0–40	mM
Model bile	NaTDC	12	mM
	Lecithin	4	mM
Lipid	Soybean oil	35	mM
	Sodium oleate	30	mM
	1-Oleoyl-rac-glycerol	15	mM

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