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Research paper

# Chitosan enhances transcellular permeability in human and rat intestine epithelium

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ABSTRACT

The intestinal epithelium regulates the transit of molecules from and into the organism. Several agents act as absorption enhancers inducing changes in both transcellular and paracellular routes. Chitosan is a non-toxic biocompatible polysaccharide widely used as dietary supplement and mucosal delivery. Chitosan triggers both the activation of intestinal epithelial cells and the release of regulatory factors relevant for its immunomodulatory activity. Yet, the interaction of chitosan with intestinal epithelial cells is poorly characterized. We studied the uptake of this polysaccharide, and we evaluated its effects in both the net water and ion movements across human and rat colon samples and the epithelial permeability. Herein, we demonstrate that chitosan increases the transcellular permeability to ions, water and protein markers in human and rat intestinal mucosa and decreases the water permeability across the paracellular pathway. These findings are relevant to understand the activity of the polysaccharide in the mucosal environment.



## 1. Introduction

The intestinal epithelium, a major area of contact with exogenous antigens such as microorganisms, foods and drugs, plays a critical role in the homeostasis of the gut [1]. Intestinal epithelial cells (IECs) regulate the transit of molecules from and into the organism. The continuous passage of molecules serves to sample the luminal content as well as to condition the mucosal immune system beneath [2,3]. Transport occurs by two possible routes: the transcellular pathway by which lipophilic drugs and small molecules permeate across cell membranes or are transported by channels, pumps and carriers buried in the plasma membrane; or the paracellular way that is regulated by tight junctions (TJs) and accounts for the transit of hydrophilic and larger molecules [4]. Several agents can act as absorption enhancers inducing changes in both transcellular and paracellular routes [5,6].

Chitosan (Ch) is a natural polysaccharide obtained by *N*-deacetylation of chitin. This non-toxic, biocompatible and biodegradable polymer has good mucoadhesive properties in acidic environments although loses its charges in neutral and basic environments as in intestine [7]. Nevertheless, Ch and derivatives have applications in controlled drug release for mucosal delivery [8]. Powder and solution formulations as well as nano- and microparticles of Ch are able to enhance systemic and mucosal immune responses to specific antigens after nasal or gut delivery [9]. Upon feeding, Ch is recovered in Peyer's patches and mesenteric lymph nodes (MLNs) inside immature dendritic cells [10]. At the inductive mucosal sites, this polysaccharide exhibits strong modulatory properties, enhancing the regulatory cytokine environment [10] and suppressing the inflammatory activity of lymphocytes [11]. The intestinal epithelium is mildly activated upon Ch feeding, with the release of regulatory factors afterward that could be relevant for the biocompatibility and biological effects of the polymer [12]. However, the early interaction of Ch with the epithelial barrier is poorly characterized. In colonic cell monolayers, Ch increases cell permeability by affecting paracellular and intracellular pathways without changing junctional morphology [13]. The contact with the epithelial lining seems to combine bioadhesion with transient widening of TJs [7]. Several studies using cell cultures or animal models have shown the uptake of Ch nanoparticles by Peyer's patches [14] and the epithelium as well [15]. As the contact with Ch may change the physiology and the permeability of the barrier, we used different experimental settings to characterize the interaction of low to moderate doses of soluble Ch with the epithelium.

## 2. Methods and materials

### 2.1. Chemicals

We used 85%-deacetylated low MW Ch (~80 kDa) (Aldrich Chemical CO., Inc., Milwaukee, WI, USA) and FITC-Ch (CarboMer,

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Inc., San Diego, CA, USA) as described [10–12]. Stock solutions of chitosan were prepared in 0.1 M acetic acid and diluted with culture medium or Ringer solution (pH 6.8–7.2) to the appropriate concentration for experiments [16].

### 2.2. Animals

We used 8–10 week-old female Wistar rats weighing 180–230 g housed and cared at the Animal Resource Facilities, in accordance with the institutional guidelines. The Animal Experimentation Ethics Committee from the Chemical Sciences Faculty approved the protocols.

### 2.3. Cell lines and purified intestinal epithelial cells

Mouse macrophage-like RAW 264.7 cells, rat intestinal epithelial IEC-6 cells, human colon cancer HT-29 cells and human differentiated Caco cells (C2BBe1), a brush border expressing cell line that forms a polarized monolayer with an apical brush border morphologically comparable to that of the human colon, were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37 °C in a 5% $CO_2$ humidified incubator. We isolated IECs from normal rats according to standard procedures [17]. Briefly, small guts were removed and all Peyer patches discarded. Small gut was rinsed in $Ca^{2+}$ and $Mg^{2+}$ free PBS and washed in PBS-dithiothreitol (200 μg/ml) to eliminate residual mucus and then shacked in RPMI 1640 (Sigma–Aldrich, Saint Louis, Missouri, USA) containing 0.03 M EDTA to remove the epithelium at 37 °C. Detached cells were washed, counted and adjusted to the desired concentrations for different studies. Purity was assessed by morphology in cytocentrifuge smears stained with May Grünwald Giemsa.

### 2.4. Cellular uptake

Cellular uptake was performed as described [18]. Briefly, mouse RAW 264.7, human C2BBe1 or rat IEC-6 was cultured at $10^6$ cells/well with or without 1 μg/ml LPS for 24 h. After pre-stimulation, cells were washed and 0.1, 1 or 10 μg/ml FITC-Ch was added for 30, 60, 90 or 120 min. Cells were removed with 0.25% trypsin–0.02% EDTA, fixed and analyzed by flow cytometry. IECs isolated from normal rats were placed in sterile 24-well tissue incubated with 0.1, 1 or 10 μg/ml FITC-Ch for 30, 60, 90 or 120 min. Then, cells were washed, fixed in 2% formaldehyde and resuspended in PBS. Untreated epithelial cells (primary IECs or cell lines) were included for intrinsic autofluorescence control. On the basis of forward and side light scatter, epithelial cells were gated in and debris and dead cells were gated out; 10,000 events were analyzed using a FACSAria flow cytometer (BD Bioscience), and data were analyzed using a WinMDI 2.8 (J. Trotter, Scripps Research Institute, La Jolla, CA). Results are presented as the percentage of FITC-positive cells.

### 2.5. Confocal imaging

IECs purified from normal rats as above were cultured with 1 or 10 μg/ml FITC-Ch during different times at 4 or 37 °C. Then, cells were washed, placed on a glass slide and mounted with Mowiol 4-88 (Calbiochem). Glass slides were allowed to cure overnight, in the dark. Confocal images were collected using an Olympus Fluoview FV300 laser scanning confocal microscope (Olympus Latin America, Miami, FL) equipped with an argon/helium/neon laser and a 60× (numerical aperture = 1.42) immersion objective (PlanApo). Single confocal sections of 0.7 μm were taken parallel to the coverslip (*xy* sections). Final images were compiled with Adobe Photoshop CS.

### 2.6. Specimen collection and preparation

Sigmoid colon tissues were obtained from patients undergoing subtotal colostomy for colon cancer. Before surgery, all patients had given their fully informed and written consent about the aims of the surgical intervention. Immediately after ablation, sections of macroscopically unaffected regions were placed in ice-cold high $K^+$ Ringer solution containing (in mM) 120 KCl; 10 $NaHCO_3$; 1.2 $MgCl_2$; 1.2 $CaCl_2$;1.2 $K_2HPO_4$; 0.2 $KH_2PO_4$; and 25 glucose to preserve the transport functions and carried immediately to the laboratory. Then, mucosa and submucosa layers were dissected from the underlying tissues and mounted in a modified Ussing chamber (1.76 $cm^2$) with two chambers, each with a mucosal and serosal compartment divided by the mounted tissue [19]. Both sides of the tissue were immediately washed and bathed with a standard Ringer solution containing (in mM) 113 NaCl; 4.5 KCl; 25 $NaHCO_3$; 1.2 $MgCl_2$; 1.2 $CaCl_2$; 1.2 $K_2HPO_4$; 0.2 $KH_2PO_4$; and 25 glucose. The serosal bath was continuously bubbled with 95% $O_2$–5% $CO_2$, and the cell temperature was kept at 37 °C by a water-jacket reservoir connected to a temperature-constant circulating pump.

Rat colon specimens were surgically obtained from male Sprague Dawley rats weighing approximately 100 g and immediately mounted on the Ussing chamber (0.94 $cm^2$). Both sides of the tissue were bathed with a Ringer solution containing (in mM) 116 NaCl; 25 $NaHCO_3$; 1.2 $MgCl_2$; 2.4 $K_2HPO_4$; 0.4 $KH_2PO_4$; 1.2 $CaCl_2$; and 25 glucose.

### 2.7. Net water flux and electrical measurements

The net transepithelial water flux ($J_w$) was measured in a modified Ussing chamber connected to a special electro-optical device [19]. Briefly, tissue was held against a nylon mesh with a hydrostatic pressure of 10 or 13 cm of $H_2O$ for human or rat colon, respectively. Water movement across the tissue was measured by displacement of a photo-opaque solution inside a glass capillary tube connected to the mucosal side of the chamber via an intermediate chamber. Displacement of the liquid meniscus corresponding to an amount of water, as small as 50 nl, moving across the tissue was detected using an electro-optical device connected to a computer. To determine hydrostatic permeability (Phydr), $J_w$ was also recorded at 20 cm of $H_2O$. Once the tissue reached steady values, Ch was added at the luminal side and data were recorded at 13 and 20 cm of $H_2O$ again. Phydr was calculated as $\Delta J_w/\Delta P$ for each condition being $\Delta P$ the transepithelial hydrostatic pressure gradient (20–13 cm of $H_2O$). The spontaneous potential difference (PD) was simultaneously recorded in other chamber across the calomel electrodes, via agar bridges placed adjacent to the epithelium under open circuit conditions. The short circuit current ($I_{sc}$) was measured by an automatic voltage clamp system that kept PD at 0 mV. Ch was added to the mucosal bath (time 0), at 0.6–1 μg/ml final concentration. Variations in $J_w$ and $I_{sc}$ were continuously recorded for at least 60 min. Because of tissue variability, data are presented at $\Delta J_w$ and $\Delta I_{sc}$, where $\Delta J_w = J_w$ at time $t - J_w$ at time 0 and $\Delta I_{sc} = I_{sc}$ at time $t - I_{sc}$ at time 0.

### 2.8. Assay for arachidonic acid release

HT-29 cells ($1 \times 10^6$/well) were labeled overnight with 0.5 μCi/ml [$^3$H]-arachidonic acid (specific activity 100 Ci/mmol; New England Nuclear, Boston, MA, USA). Non-incorporated label was eliminated by washing three times with serum-free medium containing 0.1 mg/ml albumin. Cells were stimulated 60 min later with 1 or 10 μg/ml polysaccharide in the same medium ($t = 0$). Supernatants were collected at different times, centrifuged at 1400*g* for 10 min and assayed for radioactivity by liquid scintillation counting [20]. When indicated, HT-29 cells were pre-treated

with the specific inhibitors scalaradial (4 μM), pyrrophenone (1 μM) or bromoenol lactone (5 μM) before Ch stimulation as described elsewhere [21,22].

### 2.9. Intestinal permeability assessment

The mucosal-to-serosal flux of [$^{14}$C]-inulin (0.5 μCi/ml, New England Nuclear Corp., USA) in rat colon specimens mounted in Ussing chamber was assessed as described [23]. [$^{14}$C]-inulin was added to the luminal side, and samples (200 μl) were collected from the serosal side every 15 min during 120 min after inulin addition and replaced with identical fresh Ringer buffer amount. After 60 min, 1 μg/ml Ch was added to the luminal side, and the isotope transport was evaluated for another 60 min, and the radioactivity of [$^{14}$C] was counted by scintillation counter (Beckman LS-5801).

The mucosal-to-serosal flux of type IV-A horseradish peroxidase (HRP) (Sigma, USA) in rat colon specimens mounted in Ussing chamber was assessed as previously mentioned [24]. HRP or HRP plus 1 μg/ml Ch was added to the luminal side, and serosal buffer aliquots of 200 μl were collected every 15 min during 120 min after HRP addition and replaced with identical fresh Ringer buffer amount. Samples were collected from the serosal side of the chamber in a time-dependent manner, and HRP activity was quantified in 50 μl of sample with 50 μl of TMB (3,3′,5,5′-tetramethylbenzidine) Substrate Reagent Set (BD OptEIA™, BD Biosciences Pharmingen) by a colorimetric method.

### 2.10. Intestinal loops

Rats were fasted overnight and then anesthetized for surgery with an intramuscular injection of 55 mg ketamine/kg body weight and 11 mg xilazine/kg body weight. Body temperature was kept at 37 °C with a lamp. The abdominal cavity was opened by a midline incision, and one 3-cm long small intestine segment (loop) was prepared in each rat. Loops were inoculated intraluminally with 0.2 mg/ml (final concentration) of HRP (Sigma, USA) with 1 μg/ml Ch in 200 μl final volume or PBS. After 60 min, rats were killed, and serum was collected and stored at −20 °C until the HRP evaluation of by colorimetric assay as above.

### 2.11. Transmission electron microscopy

For ultrastructure studies, sections of gut loops from diluent or Ch groups were washed with ice-cold PBS, fixed by immersion in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 h at 4 °C and then treated with 1% osmium tetroxide, dehydrated and embedded in Araldite. Thin sections cut in a Porter-Blum MT2 ultramicrotome equipped with a diamond knife were examined in a Zeiss 109 electron microscope as described previously [25].

### 2.12. Statistical analysis

Significance and differences among groups were determined by ANOVA and Student–Newman–Keuls tests. $p < 0.05$ values were considered statistically significant.

## 3. Results

### 3.1. Uptake of the polysaccharide

We used differentiated epithelial brush border C2BBe1, rat IEC-6 or mouse macrophage RAW264.7 cell lines to assess the uptake of Ch in similar conditions to the *in vivo* experiments [10–12]. Resting or LPS stimulated cells were cultured with 1 or 10 μg/ml FITC-Ch for different times. As can be seen (Fig. 1A), Ch uptake was dependent on both the dose of FITC-polysaccharide and the activation of the three cell lines studied. As expected, macrophages showed higher internalization of the polysaccharide than epithelial cells. On the other hand, at 10 μg/ml FITC-Ch, the percentage of fluorescent cells was higher for C2BBe1 than for IEC-6 epithelial cells possibly due to different interactions of Ch with each cell line. When primary rat IECs were cultured with FITC-Ch, we found a dose dependent increment both in the % of positive cells and in the mean fluorescence intensity (MFI) after 30 min incubation (Fig. 1B). Additionally, the MFI was evaluated in primary IECs cultured at 4 and 37 °C with 2.5–7 μg/ml FITC-Ch for 60 min (Fig. 1C). At 37 °C, we found width histograms without changes in MFI; at 4 °C instead, we observed small increments in MFI and narrow histograms, suggesting that at 4 °C the uptake process is inhibited. As flow cytometry does not discriminate between cellular adhesion and internalization, we performed uptake experiments at 4 and 37 °C to capture confocal images that confirm that fluorescent cells have effectively internalized the polysaccharide. The micrographs in Fig. 1D represent the optical sections ($x$–$y$ axis; $x$–$z$ axis and $y$–$z$ axis) of IECs after 1 h incubation with 10 μg/ml FITC-Ch. At 4 °C, a thin fluorescent layer coincident with the cell outline was detected, while at 37 °C, the fluorescence was observed inside the cells, in a vesicular pattern. This result supports the flow cytometry data and indicates that Ch is endocyted by IECs through an active mechanism.

### 3.2. Ch stimulates the arachidonic acid release

One possible signal associated with the Ch-epithelium interaction could be the release of arachidonic acid and its metabolites, as they participate in the gut integrity and regulate mucosal secretions. As shown in Fig. 2, 1 and 10 μg/ml Ch stimulated a fourfold increase in arachidonic acid release from the human colon cancer HT-29 cells after 4 and 6 h culture compared with unstimulated cells ($p < 0.05$). Interestingly, Ch mediated release occurred faster than the stimulated one by the inflammatory mediator TNF-α, which produced a milder discharge of this intermediate after 6 h of culture. The production of arachidonic acid involves the activity of phospholipases such as PLC, PLD or the $PLA_2$ superfamily. In our experimental condition, the pre-treatment of HT-29 cells with specific inhibitors of secreted (scalaradial), cytosolic (pyrrophenone) or $Ca^{(2+)}$ independent (bromoenol lactone) $PLA_2$ before Ch stimulation had no effect on the release of arachidonic acid (data not shown), suggesting that conventional $PLA_2$ was not involved in the mobilization of arachidonic acid induced by Ch.

### 3.3. Ch diminishes paracellular water permeability and increases short circuit current

As the arachidonic acid release can affect the epithelial physiology, we evaluated several parameters such as transepithelial water flux ($J_w$), short circuit current ($I_{sc}$) and hydrostatic pressure in rat colon samples mounted in the Ussing chamber (Fig. 3). At a hydrostatic pressure of 13 cm of $H_2O$, no changes in $J_w$ were detected after Ch addition. However, at a pressure of 20 cm (arrowheads), the increment in $J_w$ observed in the absence of Ch (left) was significantly lower after the polysaccharide adding (right) ($p < 0.05$). The result shows the ability of Ch to decrease the hydrostatic permeability of colonic tissue and to reduce the water paracellular permeability. Similar results were obtained with human colon samples (Fig. 4). In terms of $I_{sc}$, the addition of 1 μg/ml Ch produced a significant increment ($p < 0.05$), suggesting that the interaction of this polysaccharide with the epithelium affects ion transport (Fig. 3). After removing Ch, the electrophysiological parameters recovered basal values (data not shown).

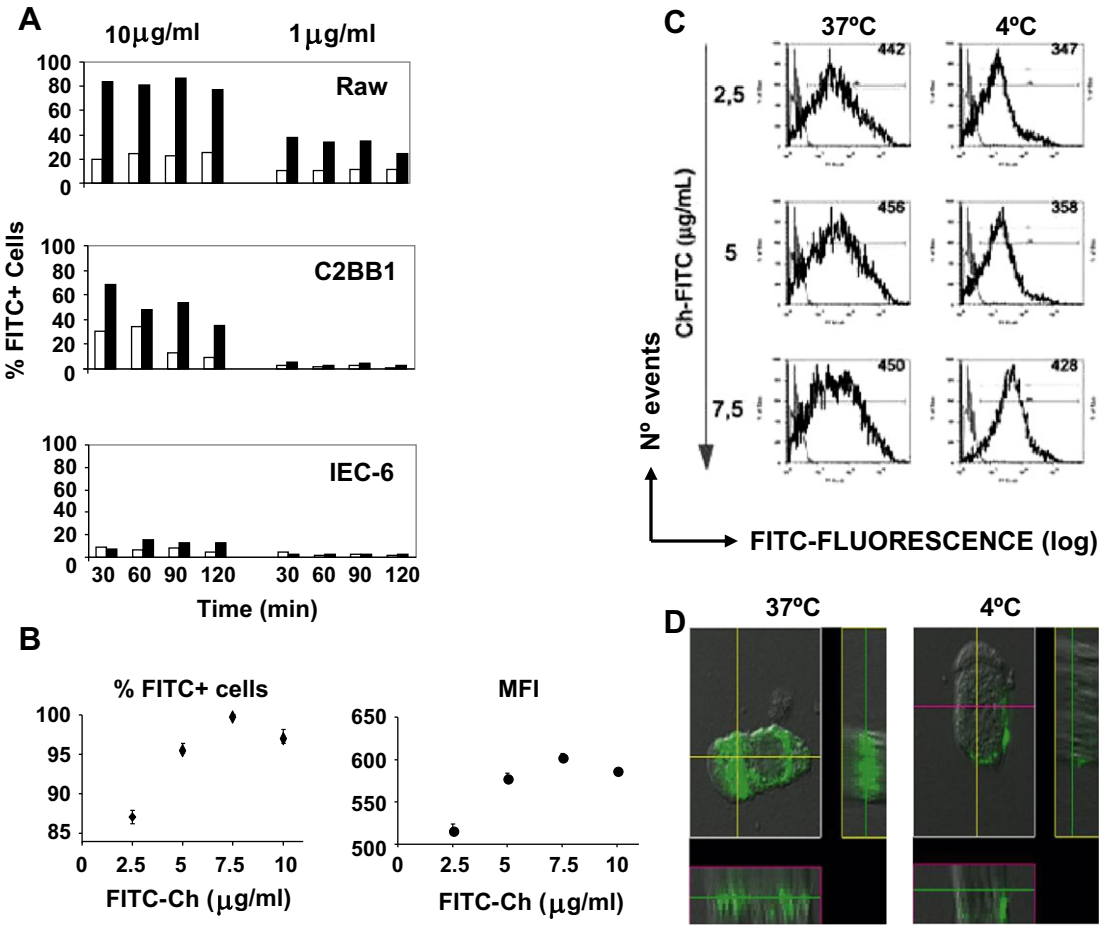


**Fig. 1.** Intestinal epithelial cells uptake the polysaccharide Ch. (A) Mouse macrophage-like RAW 264.7, human epithelial brush border differentiated Caco (C2BBe1) and rat intestinal epithelial IEC-6 cells were incubated with 1 or 10 µg/ml FITC-Ch for 0–120 min and then evaluated by flow cytometry. Cells were pre-incubated with 10 µg/ml LPS for 24 h (black bars) or medium (white bars). A representative experiment of two similar is shown. (B) Assay was performed with intestinal epithelial cells isolated from normal rats; results are presented as percentage of fluorescent positive cells and mean fluorescence intensity (MFI). (C) Intestinal epithelial cells isolated from normal rats were incubated with different doses of FITC-Ch at 4 °C and 37 °C and evaluated by flow cytometry or confocal microscopy (D). Experiments were performed two (A and D) or three times (B and C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### *3.4. Ch affects transcellular permeability*

As changes in electrophysiological parameters could be related to enhanced paracellular or transcellular permeability, we used [$^{14}$C]-inulin, a membrane-impermeant paracellular tracer and HRP, a marker of the mucosal-to-serosal flux via the transcellular way to address this possibility. As can be seen (Fig. 5), the inulin flux was similar with or without Ch addition, showing that Ch is not enhancing the paracellular permeability. In experiments with the Ussing chamber, a two- to threefold increment in HRP occurred when HRP and Ch were added together at the luminal side (Fig. 6A). The intestinal loop assay gave similar results (Fig. 6B), with increments in the enzyme activity in serum samples 1 h after the co-injection of HRP and Ch. Values were comparable to those obtained with indomethacin, a well-known permeability enhancer. When the HRP was injected into the loop without Ch (diluent group), the activity was similar to normal serum. We also evaluated the ultrastructure of TJs in colon samples from rats fed either diluent or Ch. As shown in representative microphotographs (Fig. 6C), no differences in the cytoarchitecture of the intestinal barrier were observed.

## 4. Discussion

The single-cell epithelial layer of the intestinal mucosa is integral to both the discrimination of luminal stimuli and the subsequent regulation of inflammatory responses in the intestinal microenvironment [26]. IECs interact with the underlying immune cells and take part in mucosal inflammatory responses modulating the permeability and synthesizing inflammatory mediators [27]. In this work, we demonstrated that epithelial cell lines as well as purified IECs can uptake the polysaccharide Ch. The enhancement observed with purified IECs is possibly related to the strong electrostatic interactions of Ch with mucins. In fact, Ch mucoadhesive properties involve the formation of hydrogen and ionic bonds between the positively charged amino groups of the polysaccharide and the negatively charged sialic acid residues of mucin glycoproteins [28]. In fact, good mucosal adhesion of Ch occurs due to its cationic nature, which facilitates electrostatic attraction with negatively charged mucins. At higher pH values, deprotonated amino groups can participate in hydrogen bonding with mucin along with the nonionic hydroxyl groups [29]. The role of nonionic interactions in mucosal adhesion of Ch is also confirmed by the decreased mucoadhesivity upon quaternization of amino groups [30]. Moreover, a comparative study of the uptake of Ch nanoparticles by Caco-2 and mucus-secreting MTX-E12 cells shows that the association of nanoparticles increases strongly with mucus [31]. Cellular uptake seems to occur by active endocytosis as is strongly reduced at 4 °C, and confocal imaging confirmed the internalization of soluble Ch, in agreement with a previous report using Ch nanoparticles and the human cell line derived from the respiratory epithelium A549 [32].

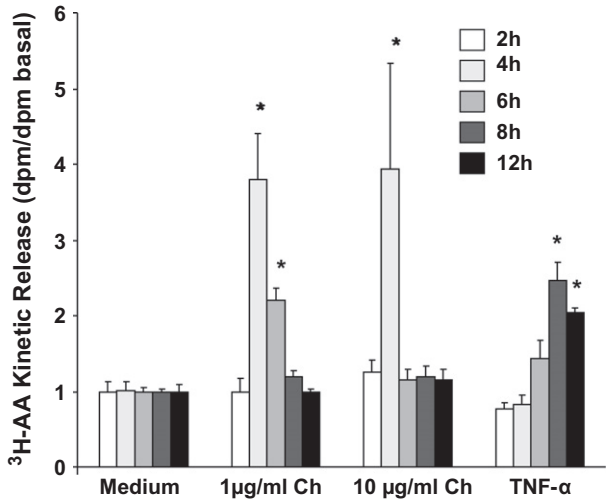


**Fig. 2.** Chitosan stimulates the mobilization of arachidonic acid. The human colorectal cancer cell line HT29 ($1 \times 10^6$) was labeled with [$^3$H]arachidonic acid and prepared for the assay as described in Section 2. Cells were treated with different stimuli ($t = 0$), and after 2 h, supernatants were removed, cleared of detached cells by centrifugation and assayed for radioactivity by liquid scintillation counting. Bars are the ratio dpm/basal dpm ± SD for each condition and time evaluated. $^*p < 0.05$ vs. medium.

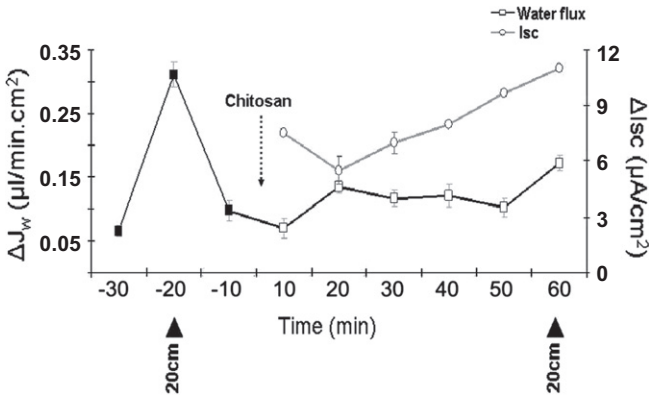


**Fig. 3.** Influence of Ch in water flux and electrical parameters. Rat colon specimens mounted in Ussing chamber were sequentially monitored for water flux changes ($J_w$, µl/min cm$^2$) and short circuit current ($I_{sc}$, µA/cm$^2$) with voltage clamped to zero. Water flux was measured in the basal state (filled squares) and during incubation with 1 µg/ml Ch (empty squares). Electrical parameters were measured during incubation with the polysaccharide (empty circles). Arrows indicate a hydrostatic pressure of 20 cm in these points; otherwise, hydrostatic pressure is 13 cm. Significance was determined by Student's $t$ test for paired data of Ch vs. basal; $^*p < 0.05$. A representative experiment of four similar is shown.

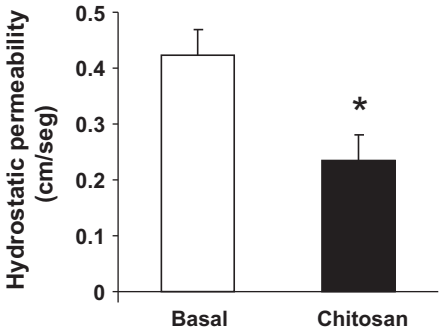


**Fig. 4.** Hydrostatic permeability in human colon samples after Ch treatment. $J_w$ was measured as described in Fig. 3. Hydroosmotic permeability was calculated as described in Section 2. Means ± SD of three samples. Experiments were performed twice. $^*p < 0.05$ Ch vs. basal.

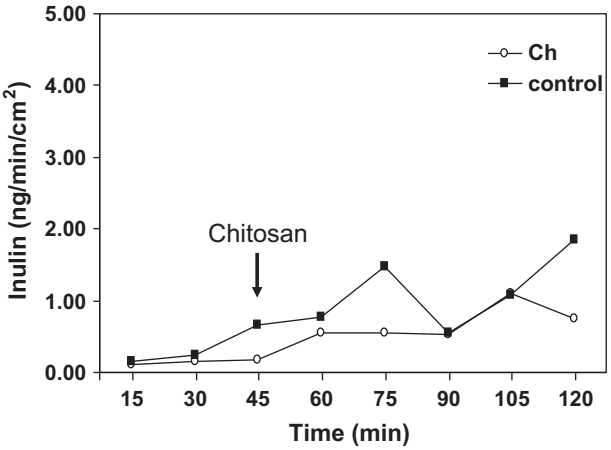


**Fig. 5.** Paracellular permeability in rat colon samples after Ch treatment. Rat colon samples were mounted in Ussing chamber as above. [$^{14}$C]-Inulin was added to the luminal side, and 200 µl aliquot of the serosal buffer was collected every 15 min during 120 min after inulin addition and replaced with identical fresh Ringer buffer amount. After 45 min, 1 µg/ml Ch was added to the luminal side. The radioactivity of [$^{14}$C] was counted by scintillation counter, and concentration was calculated. A representative of two similar experiments is shown.

Early upon contact, Ch triggers the release of arachidonic acid from human colon cells, even faster than after TNF-α stimulation. The ability of polysaccharides to elicit robust release of arachidonic acid in different leukocyte subsets and epithelial cells has been reported: zymosan triggers the arachidonic acid release from immature dendritic cells after 30 min stimulation at low concentrations [33], whereas Ch causes arachidonic acid release from macrophages, already after 5 min incubation [34]. On the other hand, LPS stimulates the release of arachidonic acid metabolites from bronchial epithelial cells as well as rat enterocyte cell line IEC 18 [35,36]. The inhibition experiments suggested that none of the conventional $PLA_2$ is involved and possibly other enzymes such as PLC plus diacylglycerol lipase could release arachidonic acid in our system. Although we have not explored this possibility yet, the PLC pathway has been described in the same cell line that we used in our study [37].

The bioactivity of arachidonic acid itself or its derived active products may affect the regulation of intestinal permeability [38], as reported in Caco-2 cell cultures [39–41]. Herein, we found no changes in the net absorptive water flux when 1 µg/ml Ch was added to the mucosal side of colonic tissues. Previous studies on intestinal barrier have shown the striking movements of electrolytes and water in both directions across the intestinal mucosa. These movements are normally balanced slightly in favor of net absorption [42]. We found a net water absorption in human and rat colon mucosa before and after addition of Ch to the mucosal side. However, Phydr was significantly lower in the presence than in the absence of Ch. It is generally accepted that Phydr gradients, in the range employed (24.2 cm water = 1 m Osm), do not cause transcellular water fluxes and the paracellular route is the pathway for the water movement [43]. Therefore, a reduced Phydr in both rat and human colon samples suggests that the TJs become tighter for water in the presence of Ch. These findings appear to be different to those previously described in Caco-2 cells. In these reports, Ch seems to act mainly on negative sites at the cell surface and TJs inducing changes in localization of proteins facilitating the widening of TJs and enhancing transport through the paracellular path-

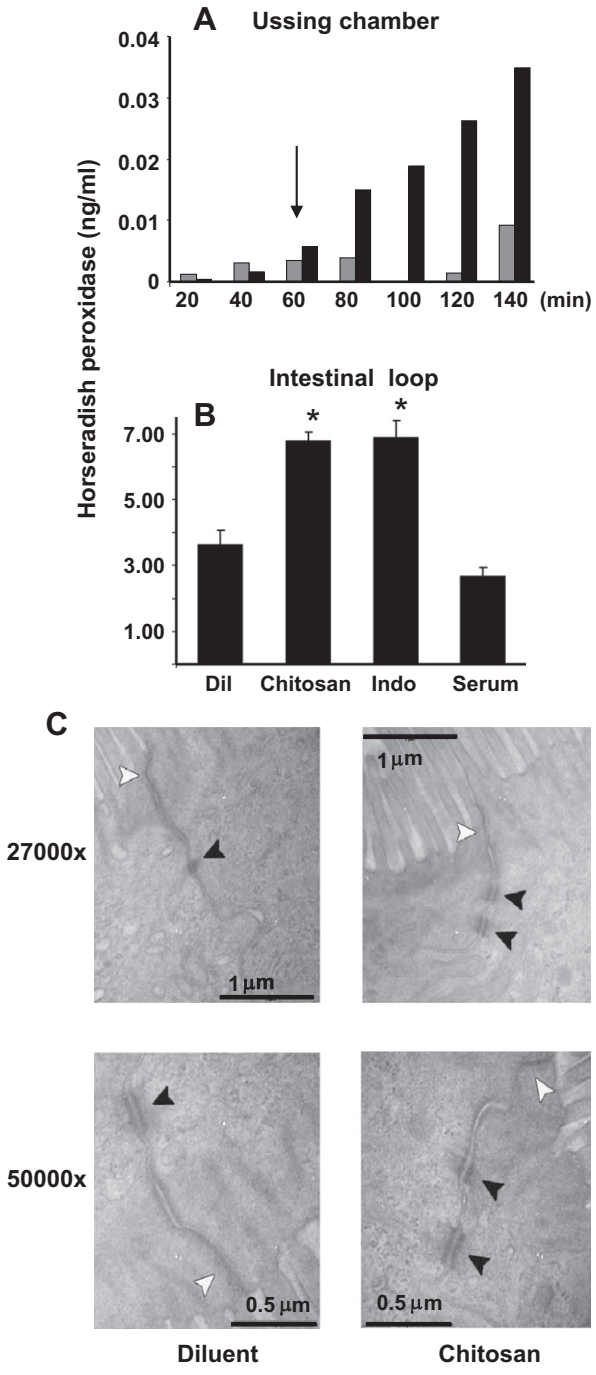


**Fig. 6.** Transcellular permeability after Ch treatment. Transcellular permeability was assessed by the flux of horseradish peroxidase (HRP) in rat colon samples mounted in Ussing chamber (A) or injected in intestinal loops (B). (A) Ch was added after 60 min (indicated by the arrow), and the HRP activity was evaluated by a colorimetric assay at the indicated times. Gray bars: HRP activity without Ch addition; black bars; HPR activity with Ch. Representative of three similar experiments. (B) Loops were prepared as described in Materials and Methods. The enzyme was injected at 0.2 mg/ml plus diluent (acetic acid), Ch or indomethacin (Indo) as positive control. After 1 h, rats were killed and blood samples were obtained. The enzyme activity was measured in serum with a colorimetric assay, and results are expressed as ng/ml. Data are means ± SD of 3–4 loops per treatment. For comparative purposes, HPR values of normal serum are included. (C) Representative samples were processed for electron microscopy after the intestinal loop assay. Bar either 1 µm (upper) or 0.5 µm (lower). White arrowheads: TJs; black arrowheads: desmosomes.

way [7,44]. It has been shown that Ch promotes the transmucosal absorption of small polar molecules, peptide and drugs as well as [$^{14}$C]-mannitol flux, a marker for paracellular routes [45]. Herein, in rat colon using 1 µg/ml Ch, we have not observed an increase in [$^{14}$C]-inulin flux due to Ch. Indeed, in rat colon using 1 µg/ml Ch, we have not observed an increase in [$^{14}$C]-inulin flux due to Ch. Discrepancies may be attributed to the differences in the experimental settings used as the intestinal mucosa has a highly folded effective absorptive area and a heterogeneous cell population that may not be represented in the flat layer epithelium in cell models [46]. Moreover, salt form, MW and amount of Ch influence the permeation-enhancing effects [47,48]. Herein, inulin passage to serosal side was 0.1% after 2 h of Ch treatment, indicating no enhancement of transport across TJs. Ranaldi et al. [49] reported similar results in Caco-2 cell line when Ch doses were twofold higher than those used in our experiments. According to their results, TJs structure remains unmodified up to 2 µg/ml.

We found a significant increase in $I_{sc}$ after Ch addition; $I_{sc}$ is a parameter frequently employed to evaluate ionic transport across epithelial barriers. In the absence of a saline gradient between mucosal and serosal sides, $I_{sc}$ represents both the active $Na^+$ absorption and $HCO_3^-$ and $Cl^-$ secretion in human colon [50]. Active ion absorption and secretion resulting in a net water transfer across the barrier is driven by a salt transport-generated osmotic gradient. However, the increase in $I_{sc}$ in the presence of Ch was not accompanied by an increase in $J_w$. If we assume that the $I_{sc}$ is mainly the result of the electrogenic $Na^+$ absorption coupled to the water transfer across the transcellular route, we could expect an increase in $J_w$ associated with an increase in $I_{sc}$ [42]. The fact that $J_w$ remains constant may result of a balance between the increase in water transport across the transcellular pathway and a decrease in water transport across the paracellular pathway in the presence of Ch.

Together, the transcellular enhancing permeability of Ch seems dependent on the increment of the absorptive flux that could be associated with the uptake of the polysaccharide. In agreement, binding of Ch to Caco-2 cells seems to precede absorption enhancement [44]. Herein, Ch increased the HRP uptake as we detected a significant increment of the enzyme activity in the blood. These results are in good accordance with previous results showing that the incubation of Caco-2 cells with 0.05–0.1% Ch increased HRP permeability [48].

Recently, we demonstrated that soluble Ch stimulates *in vivo* biochemical events at the epithelial lining that could be relevant for the biocompatibility and immunomodulatory effect of this polysaccharide [12]. Mainly, this mucoadhesive polymer mildly activates IECs and enhances the release of IL-10, IL-6 and TGF-β, the expression of several chemokines and the activity of the arginase pathway. Together, the signals elicited at the intestinal epithelium by the single administration of this cationic polymer seem to reinforce the non-inflammatory microenvironment. Herein, we show that the interaction occurs with mobilization of arachidonic acid and increment in $I_{sc}$ possibly by rise of $Na^+$ absorption. Moreover, the active uptake of the polysaccharide by IECs enhances the transcellular permeability of a co-administered protein. In agreement, the appearance of large vacuoles in Caco-2 cells after 30 min of Ch treatment demonstrates that under certain conditions Ch can affect paracellular and intracellular pathways [7]. Considering similar experimental conditions, the amount of protein co-administered with Ch that crosses the epithelial lining could be higher – or less degraded – and therefore the biological response elicited stronger. Supporting this hypothesis, we found that Ch improves the oral tolerance to the articular antigen type II collagen [51] and others observed higher levels of specific IgA after vaccination with soluble Ch [52,53]. In addition to the transient widening of TJs and the increment of the paracellular flux, other mechanisms should be considered in order to disclose the mucosal activity of this cationic polysaccharide.

## 5. Conclusions

Herein, we demonstrated the ability of the mucoadhesive polysaccharide Ch to trigger the release on arachidonic acid in IECs, to increase the transcellular permeability to ions and water in both human and rat intestine mucosa and to decrease the water permeability across the paracellular pathway. The effective uptake of the polysaccharide is associated with the transcellular internalization of the protein HRP. Our findings may help to disclose the mechanism of mucosal activity of this cationic polysaccharide.

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