Research paper

Exposure to common food additive carrageenan leads to reduced sulfatase activity and increase in sulfated glycosaminoglycans in human epithelial cells

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\section*{1. Introduction}

The common food additive, carrageenan, is consumed in the average diet in sufficient quantities to have biological effects. In contrast to the glycoside digoxin, which is generally prescribed in doses of 0.25 mg daily, average daily ingestion of carrageenan in the typical diet is estimated to be 250 mg/day \([1,2]\). Individuals who consume several carrageenan-containing foods may ingest several grams of carrageenan per day \([3,4]\). Carrageenan is found in a wide range of processed foods, including ice cream, whipped cream, infant formula, deli meats, sour cream, puddings, soy milk, yogurt, and dietary supplements. Carrageenan is also used in pharmaceuticals as an excipient, and in room air fresheners, cosmetics, and pet foods, due to its ability to improve the texture and solubility of ingredients. The Joint Expert Committee (Food and Agriculture Organization of the UN and the WHO) on Food Additives has recommended that carrageenan be excluded from infant formula and that current intake of carrageenan in the diet be re-evaluated \([5]\).

Three major types of carrageenan are used in food products. These include lambda (\(\lambda\))-carrageenan which is the most highly sulfated, with sulfate groups at \(\mathrm{C}2\) and \(\mathrm{C}2, \mathrm{C}6\); kappa (\(\kappa\))-carrageenan with sulfate groups at \(\mathrm{C}4\) of the alpha linked galactose and \(\mathrm{C}3\)-\(\mathrm{C}6\) epimerization of the beta-linked galactose; and iota (\(\iota\))-carrageenan that resembles \(\kappa\)-carrageenan, but with the addition of a second sulfate group at \(\mathrm{C}2\) of the \(\beta\)-1,4-linked galactose (Fig. 1). \(\lambda\)-carrageenan is the most soluble and has the lowest gel-forming ability of the carrageenans, while \(\kappa\)- and \(\iota\)-carrageenans readily form gels in association with potassium or calcium ions, respectively \([6,7]\).

In thousands of experiments, carrageenans have been used to induce inflammation, since inflammation is a predictable effect of exposure to carrageenan in animal and cell-based models. For the most part, these experiments were designed to test the effectiveness of anti-inflammatory agents or to study the mediators of
inflammation [8]. In recent experiments, we have identified specific mechanisms by which carrageenan causes inflammation, and have demonstrated activation of an innate immune pathway of inflammation and a reactive-oxygen species (ROS)-mediated pathway [9–12]. The innate immune pathway is mediated by toll-like receptor (TLR)-4 and B-cell leukemia lymphoma (BCL)10, leading to increased Interleukin (IL)-8 secretion by both canonical and non-canonical pathways of NF-κB activation. The ROS-mediated pathway of inflammation does not involve TLR4-BCL10, but requires Hsp27 and IkB-kinase (IKK), leading to increased phosphorylation of IkBz, and thereby enabling the nuclear translocation of NF-κB. Enzymes that hydrolyze the galactosidic bonds of carrageenan affect the extent of activation of the innate immune pathway, with κ-carrageenan-induced inflammation reduced by exposure to α→1,3,6-galactosidase and increased by κ-carrageenase [13]. Carrageenan-induced ROS-mediated inflammation was reduced following exposure of κ-carrageenan to recombinant human arylsulfatase B (ARSB), suggesting that the presence of sulfate groups on carrageenan contributes to ROS activation [14].

In the current study, we assess the effect of carrageenan exposure on sulfatase activity of human epithelial cells in culture and determine changes in GAG content and disaccharide composition and present these findings to better understand how carrageenan exposure impacts upon the endogenous GAGs.

2. Methods and materials

2.1. Cell culture

Several intestinal epithelial cell lines were grown in cell culture, including NCM460 (INCELL, San Antonio, TX) [15], T84 (ATCC), CaCO2 (ATCC), rat ileal epithelial cells (ATCC), mouse intestinal epithelial cells, and primary human colonocytes obtained at the time of colonic surgery, in accord with an IRB-approved protocol at the University of Illinois at Chicago. Mammary cell lines included: MCF-7 (ATCC), MCF-10A (ATCC), T47D (ATCC), HCC1937 (ATCC), and primary myoepithelial cells and primary epithelial cells, obtained from reduction mammoplasty in accord with an IRB-approved protocol. The methods for culture of these cells were reported previously and were those that were recommended [16,17]. Cells were grown under established conditions, including 37 °C, 5% CO2, and 95% humidity, with media changes at 2–3 day intervals.

2.2. Measurement of activity of sulfatases

Activity of several sulfatases was measured in NCM460 and MCF-7 cell homogenates at 80–90% confluence after 4–6 days in culture, as indicated in Tables 1 and 2 and in Results 3.1 and 3.2. Cells were exposed to α-, κ-, or i-carrageenan (1 µg/ml) (Fig. 1), and activity was compared to unexposed cells grown simultaneously under the same conditions. Methods used for assay of N-acetylgalactosamine-4-sulfatase (Arylsulfatase B, ARSB), galactose-6-sulfatase (GALNS), steroid sulfatase (STS), iduronate-2-sulfatase (IDS), and arylsulfatase A (ARSA) were published previously [16]. Cell homogenates were prepared and assays were performed using at least triplicate biological samples with technical replicates of each measurement.

ARSB activity assay was performed by a fluorometric assay with 20 µl of cell homogenate, 80 µl of assay buffer (0.05 M Na-acetate buffer, pH 5.6), and 100 µl of substrate (5 mM 4-MUS in assay buffer) in wells of a microplate. The microplate was incubated for 30 min at 37 °C. The reaction was stopped by adding 150 µl of stop buffer (sucine-Carbonate buffer, pH 10.7), and fluorescence was measured at 360 nm (excitation), and 465 nm (emission) [FLUOstar, BMG Labtech, Inc., Gary, NC]. ARSB activity units are nmol/mg protein/hour, and were derived from a standard curve prepared with known quantities of 4-methylumbiliferyl at pH 5.6.

Substrates for determinations of activity of galactose-6-sulfatase (GALNS) and iduronate-2-sulfatase (IDS) were obtained (Moscard Substrates, Rotterdam, The Netherlands), and the assays were performed in accord with previously published protocols [18,19]. GALNS assay was performed with 5 µl cell homogenate made in ddH2O by sonication with metal tip combined with 5 µl 0.2% heat-inactivated BSA (or 10 µl of 0.2% heat-inactivated BSA for blank) and 20 µl of substrate [10 mM 4-methylumbiliferyl-β-D-galactoside-6-sulfateNH4 (MU-βGal-6S)] in substrate buffer [0.1 M sodium acetate/0.1 M acetic acid at pH 4.3 with 0.1 M NaCl, 5 mM Pb-acetate (1.9 mg/ml) and 0.02% Na-azide] in wells of a microtiter plate. The plate was sealed, and incubated for 17 h at 37 °C. Next, 5 µl 0.9 M Na-Phosphate buffer at pH 4.3 with 0.02% Na-azide was added, as well as 10 µl of 10 U β-D-Galactosidase (Sigma) /ml 0.2% heat-inactivated BSA. Reactants were incubated for 2 h at 37 °C, and then 200 µl of stop buffer [0.5 M NaHCO3/0.5 M Na2CO3 at pH 10.7 with 0.025% Triton-X-100] was added. Fluorescence readings were taken at 360 nm and 465 nm. GALNS activity is expressed as nmol/mg protein/hour.

For IDS activity, 10 µl of cell homogenate made in water by sonification with a metal tip [or 10 µl 0.2% heat-inactivated bovine serum albumin (BSA) for the blank] was combined with 20 µl of substrate in substrate buffer (1.25 mM 4-MUS in 0.1 M Na-acetate/0.1 M acetic acid at pH 5.0 with 10 mM Pb-acetate). Cell homogenate, substrate, and substrate buffer were incubated 37 °C × 4 h. After 4 h, 40 µl of 0.4 M Na-Phosphate/0.2 M citrate buffer at pH 4.5 and 0.02% Na-azide were added, as well as 10 µl of LEBT (lysosomal enzymes purified from bovine testis), followed by incubation for 24 h at 37 °C. Next, 200 µl of stop buffer consisting of 0.5 M NaHCO3/Na2CO3 at pH 10.7 with 0.025% Triton-X-100 was added, and readings were taken at 360 nm and 465 nm. IDS activity is expressed as nmol/mg protein/hour.

ARSA activity was determined by a spectrophotometric technique using p-nitrocatechol sulfate (NCS) as substrate, and based on the retention of ARSA activity at low temperature, in contrast to ARSB activity [20,21]. Pre-chilled reagents were used, and cells were homogenized in ddH2O by sonication. Cell homogenates were centrifuged for 10 min at 5000 g at 4 °C, and the supernatant was
used for the enzyme activity assay. Cell extracts were incubated in a total volume of 200 μl 0.5 M Na-acetate buffer at pH 5.0 with 200 μl of 10 mM p-NCS in an ice-bath. After 17 h, the reaction was stopped by adding 600 μl of 1 M NaOH, and the reaction mixture was centrifuged for 5 min to remove precipitated material. The optical density of the clear supernatant was measured at 516 nm in a Beckman spectrophotometer. ARSA activity is reported as nmol/mg protein/hour.

**Table 1**

Sulfatase activity in intestinal epithelial cells following exposure to κ-carrageenan x 4 days A.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>ARSBa</th>
<th>ARSAa</th>
<th>STSb</th>
<th>GALNSd</th>
<th>IDSe</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCM460</td>
<td>Control</td>
<td>125.5 (1.1)</td>
<td>107.6 (8.8)</td>
<td>26.8 (0.5)</td>
<td>8.6 (0.3)</td>
<td>11.1 (0.2)</td>
</tr>
<tr>
<td></td>
<td>κ-Carrageenan</td>
<td>75.2 (4.6)**</td>
<td>66.9 (5.9)**</td>
<td>13.9 (0.2)**</td>
<td>5.2 (0.002)**</td>
<td>8.4 (0.04)**</td>
</tr>
<tr>
<td></td>
<td>x-Carrageenan</td>
<td>83.7 (1.5)**</td>
<td>75.6 (7.0)**</td>
<td>16.2 (0.9)**</td>
<td>6.9 (0.5)**</td>
<td>8.6 (0.04)**</td>
</tr>
<tr>
<td></td>
<td>CaCO2</td>
<td>90.3 (3.8)**</td>
<td>79.4 (6.9)**</td>
<td>16.7 (2.4)**</td>
<td>7.2 (2.5)**</td>
<td>8.6 (0.05)**</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>143.4 (10.3)</td>
<td>97.9 (3.7)</td>
<td>22.9 (0.3)</td>
<td>5.4 (0.2)</td>
<td>5.9 (0.1)</td>
</tr>
<tr>
<td></td>
<td>κ-Carrageenan</td>
<td>107.5 (4.9)**</td>
<td>61.8 (5.0)**</td>
<td>16.8 (0.3)**</td>
<td>3.5 (0.1)**</td>
<td>3.0 (0.1)**</td>
</tr>
<tr>
<td></td>
<td>Colonocytes</td>
<td>113.6 (2.9)</td>
<td>141.0 (9.0)</td>
<td>28.0 (0.6)</td>
<td>9.3 (0.4)</td>
<td>12.1 (0.4)</td>
</tr>
<tr>
<td></td>
<td>T84</td>
<td>60.0 (3.8)</td>
<td>52.5 (4.8)</td>
<td>20.1 (1.2)</td>
<td>6.3 (0.3)</td>
<td>12.7 (0.6)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>12.8 (1.4)**</td>
<td>37.9 (2.8)</td>
<td>12.5 (0.5)**</td>
<td>4.2 (0.2)**</td>
<td>8.7 (0.1)**</td>
</tr>
</tbody>
</table>

**Table 2**

Sulfatase activity in mammary cells after exposure to κ-carrageenan x 6 days.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>ARSBa</th>
<th>ARSAa</th>
<th>STSb</th>
<th>GALNSd</th>
<th>IDSe</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>control</td>
<td>53.4 (1.8)</td>
<td>85.4 (4.6)</td>
<td>14.2 (0.7)</td>
<td>3.7 (0.3)</td>
<td>13.4 (1.3)</td>
</tr>
<tr>
<td></td>
<td>κ-carrageenan</td>
<td>31.2 (0.9)**</td>
<td>60.1 (1.0)**</td>
<td>6.1 (0.5)**</td>
<td>2.4 (0.9)**</td>
<td>7.2 (0.2)**</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>control</td>
<td>74.8 (2.8)</td>
<td>57.9 (1.6)</td>
<td>62.4 (3.6)</td>
<td>4.1 (0.2)</td>
<td>16.3 (0.5)</td>
</tr>
<tr>
<td></td>
<td>κ-carrageenan</td>
<td>22.4 (1.7)**</td>
<td>29.5 (0.9)**</td>
<td>42.7 (4.4)**</td>
<td>1.8 (0.1)**</td>
<td>11.2 (1.0)**</td>
</tr>
<tr>
<td>MECc</td>
<td>control</td>
<td>412.4 (18.8)</td>
<td>262.5 (2.1)</td>
<td>81.1 (3.9)</td>
<td>5.6 (0.2)</td>
<td>13.3 (0.2)</td>
</tr>
<tr>
<td></td>
<td>κ-carrageenan</td>
<td>177.8 (15.0)**</td>
<td>117.8 (1.7)**</td>
<td>38.7 (0.1)**</td>
<td>2.3 (0.03)**</td>
<td>8.5 (0.2)**</td>
</tr>
<tr>
<td>T47D</td>
<td>control</td>
<td>47.4 (0.9)</td>
<td>86.8 (0.2)</td>
<td>14.5 (0.2)</td>
<td>4.6 (0.1)</td>
<td>16.9 (0.2)</td>
</tr>
<tr>
<td></td>
<td>κ-carrageenan</td>
<td>20.4 (0.5)**</td>
<td>62.7 (1.7)**</td>
<td>9.4 (0.2)**</td>
<td>2.8 (0.03)**</td>
<td>8.8 (0.1)**</td>
</tr>
<tr>
<td>HCC1937</td>
<td>control</td>
<td>735 (3.9)</td>
<td>121.3 (6.3)</td>
<td>25.2 (0.6)</td>
<td>4.4 (0.1)</td>
<td>17.3 (0.03)</td>
</tr>
<tr>
<td></td>
<td>κ-carrageenan</td>
<td>449 (1.7)**</td>
<td>96.6 (4.4)**</td>
<td>15.0 (0.2)**</td>
<td>2.4 (0.01)**</td>
<td>10.0 (0.4)**</td>
</tr>
<tr>
<td>ECe</td>
<td>control</td>
<td>2333 (10.1)</td>
<td>ND</td>
<td>62.8 (3.3)</td>
<td>8.4 (0.3)</td>
<td>13.1 (0.1)</td>
</tr>
<tr>
<td></td>
<td>κ-carrageenan</td>
<td>162.7 (3.8)**</td>
<td>ND</td>
<td>39.4 (0.9)**</td>
<td>6.2 (0.1)**</td>
<td>7.7 (0.4)**</td>
</tr>
</tbody>
</table>

*** for p < 0.001; ** for p < 0.01 for difference following carrageenan exposure.

a ARSB = arylsulfatase B.
b ARSA = arylsulfatase A.
c STS = steroid sulfatase.
d GALNS = galactose-6-sulfatase.
e IDS = iduronate sulfatase.
f MEC = primary mammary myoepithelial cells.
g EC = primary mammary epithelial cells.
with 10 mM 4-MU, 10 mM lead acetate, and 50 mM Hepes at pH 8.0 yielding a total volume of 30 μl. The mixture was incubated for 2 h at 37 °C. 100 μl of 0.5 M Na₂CO₃/NaHCO₃ was used to stop the reaction, and fluorescence was measured, the OD was correlated with known concentrations of 4-MU at pH 8.0, and the activity expressed as nmol/mg protein/h.

2.3. Cell-based ELISA and cDNA microarray of sulfatases

Cell-based ELISA is designed as a microplate-based Western blot (Active Motif, Carlsbad, CA), and was used to detect the protein content of ARSB, ARSA, GALNS, and STS in NCM460 and T84 cells after exposure to κ-carrageenan for 48 h [10]. Activity at 48 h was determined by the procedures described above and were within the standard deviation of the activity at 4 days that is reported in Table 1 for these enzymes. Antibodies used for detection were ARSB rabbit polyclonal antibody (1:250; Open Biosystems, Huntsville, AL), GALNS rabbit polyclonal antibody (1:250; Open Biosystems, Huntsville, AL), STS mouse monoclonal (2 μg/ml, Abcam, Cambridge, MA) and ARSA mouse monoclonal (4 μg/ml; R&D, St. Paul, MN). Rabbit or mouse IgG was used for control experiments. For the cell-based ELISAs, NCM460 or T84 cells were seeded at densities of 5000, 50000, and 100000 cells per well in a 96-well plate with n = 5 for each of the determinations. After 48 h, the media were aspirated, the cells were washed with PBS, and then fixed +20 min at room temperature with 100 μl of formaldehyde 4% in PBS. The formaldehyde was removed and cells were washed with wash buffer (0.1% Triton-X-100 in PBS) 200 μl three times, wash buffer was aspirated, and cells were incubated with quenching buffer (1% formaldehyde) for 20 min at room temperature, washed with wash buffer three times, and the secondary antibody (100 μl) was added to each well, and the plate was incubated for 30 min at room temperature. The cell samples (in 1 ml water) were individually subjected to proteolysis at 55 °C with 10% of actinase E (20 mg/ml) for 20 h. After proteolysis, dry urea and dry CHAPS were added to each sample (2 wt% in CHAPS and 8 M in urea). Particles were removed from the resulting solutions by passing each through a syringe filter containing a 0.22 μm membrane. A Vivapure MINI Q H spin column was prepared by equilibrating with 200 μl of 8 M urea containing 100 μl of 15 M NaCl. GAGs were subjected to centrifugal Filter Units YM-3 was from Millipore Company (Bedford, MA, USA). Unsaturated disaccharide standards of CS/DS (ΔDi-0S: ΔUA-GalNAc, ΔDi-4S: ΔUA-GalNAc4S, ΔDi-6S: ΔUA-GalNAc6S, ΔDi-2S: ΔUA2S-GalNAc, ΔDi-disS: ΔUA2S-GalNAc4S, ΔDi-triS: ΔUA2S-GalNAc4S6S) and unsaturated disaccharides standards of HP/HS (ΔDi-0S: ΔUA-GlcNAc, ΔDi-NS: ΔUA-GlcNS, ΔDi-6S: ΔUA-GlcNAc6S, ΔDi-triS: ΔUA2S-GlcNAc6S, ΔDi-triS: ΔUA2S-GlcNS6S) were obtained from Seikagaku Corporation (Japan). All other chemicals were of reagent grade.

The molecular weights (MW) of the CS disaccharides are: Di-0S (UA-GalNAc) 379; Di-4S(UA-GalNAc4S) 459; Di-6S(UA-GalNAc6S) 459; Di-2S(UA2S-GalNAc) 539; Di-disS(UA2S-GalNAc4S) 539; Di-triS(UA2S-GalNAc4S6S) 637. The MW of the HS disaccharides are: Di-0S(UA-GlcNAc) 379; Di-NS(UA-GlcNS) 417; Di-6S(UA-GlcNAc6S) 458; Di-2S(UA2S-GlcNAc) 458; Di-2SNS(UA2S-GlcNS) 497; Di-NS6S(UA-GlcNS6S) 497; Di-2S6S(UA2S-GlcNAc6S) 537; and Di-triS(UA2S-GlcNAc6S) 576. The molecular weights of the carrageenan disaccharides are different than those of the GAGs disaccharides. The carrageenan-derived disaccharides consist of 3,6-anhydro-galactose(A-Unit) and galactose(G-Unit) or two galactose (G-unit). The MW of the carrageenan-derived disaccharides are: kappa-carra-disaccharide (A-G4S) 404; iota-carra-disaccharide (A2S-G4S) 484; and lambda-carra-disaccharide (G4S-G2S6S) 582.

2.5. Isolation and purification of GAGs from cells

The cell samples (in 1 ml water) were individually subjected to proteolysis at 55 °C with 10% of actinase E (20 mg/ml) for 20 h. After proteolysis, dry urea and dry CHAPS were added to each sample (2 wt% in CHAPS and 8 M in urea). Particles were removed from the resulting solutions by passing each through a syringe filter containing a 0.22 μm membrane. A Vivapure MINI Q H spin column was prepared by equilibrating with 200 μl of 8 M urea containing 2% CHAPS (pH 8.3). The clarified filtered samples were loaded onto and run through the Vivapure MINI Q H spin columns under centrifugal force (2000 × g). The columns were first washed with 200 μl of 8 M urea containing 2% CHAPS at pH 8.3. The columns were then washed five-times with 200 μl of 200 mM NaCl. GAGs were released from the spin column by washing three times with 50 μl of 16% NaCl. GAGs were desalted with a Microcon Centrifugal Filter Units YM-3 (3000 MWCO) spin column. The GAGs were freeze-dried for future use.

2.6. Quantification of glycosaminoglycans

The isolated glycosaminoglycans (GAGs) were subjected to carbazole assay to quantify the amount of GAGs in each sample using heparan sulfate as standard. A standard curve of the heparan sulfate gave the equation y = 0.1065x + 0.0498, R² = 0.9886. The amount of GAGs was determined in samples of 10² cells treated with carrageenan for 4 days. The carbazole assay measures the hexuronic containing GAGs, including unsulfated GAGs (e.g. hyaluronan) and disaccharides, but not keratan sulfate or its digests. The total sulfated GAGs, including chondroitin-4-sulfate (CS), chondroitin-6-sulfate, keratan sulfate, dermatan sulfate, heparan
sulfate, and heparin, but not including disaccharides, were measured in cell lysates by sulfated GAG assay (Blyscan™, Biocolor Ltd, Newtownabbey, Northern Ireland). The cationic dye 1,9-dimethylmethylen blue reacts with the sulfated GAG, producing an insoluble dye-GAG complex, and the sGAG content is determined by the amount of dye recovered from the test sample following exposure to Blyscan Dissociation Reagent. The ratio of the dye recovered from the test sample (mg protein/g) had signiﬁcantly lower baseline ARSB, ARSA, GALNS, and STS activity than the other human colonic cell lines, and declined further carrageenan exposure. IDS activity in the T84 cells was similar to the activity in the NCM460 cells and primary colonocytes and greater than in the CaCO2 cells, in contrast to the ARSB activity which was highest in the CaCO2 cells and least in the T84 cells. All of the activity measurements declined following carrageenan exposure.

In the NCM460 cell line, which was derived from normal colonic epithelium, the effects of exposure to kappa and iota carrageenan were compared to effects of lambda-carrageenan (1 μg/ml x 4 d) (Table 1). λ-carrageenan had the greatest inhibitory effect on activity of the panel of sulfatase enzymes, and i-carrageenan showed the least inhibition. Similarly, in the T84 cells, the inhibitory effect of λ-carrageenan on ARSB activity was greatest, with activity declining from 60.0 ± 3.8 to 32.8 ± 1.4 nmol/mg protein/h (p < 0.001), and the effect of i-carrageenan was least, declining to 80% of baseline (48.0 ± 1.6 nmol/mg protein/h; p < 0.001). The differences in structure of λ-κ- and i-carrageenans are depicted in Fig. 1.

In addition to the effects on the sulfatases that remove O-sulfo side-chains, carrageenan modulated the activity of the sulfatase enzymes signifi- cantly (p < 0.001 or p < 0.01), with the least inhibition. Similarly, in the NCM460 and T84 cells, the inhibitory activity of λ-carrageenan was greatest, with activity declining from 3.8 to 1.4 nmol/mg protein/h (p < 0.001), which removes the O-sulfo group from internal sites in GAG chains, and of heparan sulfamidase (4.6 ± 0.4 to 3.0 ± 0.2 nmol/mg protein/h; p < 0.001). In contrast to marked declines in activity assays, no signifi- cant differences in the protein content of ARSB, ARSA, GALNS, or STS in the NCM460 or T84 cells were detected by the cell-based ELISA assays following exposure to λ-carrageenan x 48 h. Similarly, expression of sulfatases was not significantly modified in a cDNA microarray to be signiﬁcantly changed.

3.2. Sulfatase activity in human mammary cells following carrageenan

Consistent with the observed effects of carrageenan exposure on sulfatase activity in the human colonic epithelial cells, sulfatase activity declined significantly in several human mammary cell lines, including MCF-7, MCF-10A, T47D, HCC1937, as well as primary myoepithelial and epithelial cells (Table 2) (p < 0.001) following exposure to λ-carrageenan (1 μg/ml x 6 days). ARSB, ARSA, STS, GALNS, and IDS all declined following exposure to λ-carrageenan (p < 0.001). As reported previously, baseline activity of ARSB, ARSA, STS, and GALNS was higher in the normal, primary epithelial and myoepithelial cells than in the malignant, metastatic cell lines (MCF-7 and T47D), and sulfatase activity in the MCF-10A...
disaccharide analysis, three main types of disaccharides (ΔDi-0S, ΔDi-6S and ΔDi-4S) were detected in all samples (Fig. 2A; Table 4). Similar percentages of ΔDi-6S and ΔDi-4S were present in all samples: control (22.7%: 72.8%); κ-carrageenan (20.0%: 70.3%); τ-carrageenan (20.1%: 71.2%); and λ-carrageenan (21.2%: 71.1%). In the zoom in figure (Fig. 2B), small amounts of di-sulfated disaccharides (ΔDi-diSD and ΔDi-diSE) were only detected in the GAG samples from cells exposed to carrageenan. In contrast, these disaccharides were not present in the unexposed control cell sample.

3.4.2. HP/HS-derived disaccharides

A new UPLC/MS method was used to analyze the HP/HS disaccharide composition of GAGs from the NCM460 cells. This method is particularly useful in the analysis of small amounts of biological samples or samples having very low amounts of GAG. For the HS/HP disaccharide analysis, marked differences between carrageenan-exposed samples and unexposed control were found (Fig. 3). First, the cultured cells (not exposed to carrageenans) had 10–100 fold lower HS/HP levels than the samples exposed to carrageenan, as indicated by the noisy baseline and low intensity peaks. ΔDi-TrIS and ΔDi-0S were the only assignable peaks in the untreated sample. In contrast, three other types of disaccharides (ΔDi-6S, ΔDi-256S, and ΔDi-NS) were detected in all of the carrageenan-exposed samples. The disaccharide composition (Table 5) again

The carbazole assay detects sulfated GAG, uronic acid containing glycosaminoglycans and oligosaccharides, including hyaluronan, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparin, heparan sulfate, and disaccharides, but not keratan sulfate or carrageenan. The 1,9-dimethylmethylene blue assay is used to detect sulfated GAG, but does not detect disaccharides, carrageenan, hyaluronan, or other unsulfated oligosaccharides.

For p < 0.05, ** for p ≤ 0.01, *** for p ≤ 0.001.

Table 3

<table>
<thead>
<tr>
<th>A</th>
<th>Control</th>
<th>κ-Carrageenan</th>
<th>i-Carrageenan</th>
<th>λ-Carrageenan</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAGs (μg)</td>
<td>4.2</td>
<td>135.5</td>
<td>192.3</td>
<td>141.2</td>
</tr>
<tr>
<td>Cell Number</td>
<td>10⁷</td>
<td>10⁷</td>
<td>10⁷</td>
<td>10⁷</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>GAGs/μg/mg protein ± (S.D.)</th>
<th>NCM460 cells</th>
<th>T84 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>14.6 (0.4)</td>
<td>19.1 (0.9)</td>
</tr>
<tr>
<td>κ-Carrageenan</td>
<td>20.4 (1.2)**</td>
<td>25.7 (0.3)**</td>
</tr>
<tr>
<td>i-Carrageenan</td>
<td>17.9 (0.6)**</td>
<td>23.8 (0.7)**</td>
</tr>
<tr>
<td>λ-Carrageenan</td>
<td>16.4 (0.6)*</td>
<td>21.9 (1.4)*</td>
</tr>
</tbody>
</table>

The carbazole assay detects sulfated GAG, uronic acid containing glycosaminoglycans and oligosaccharides, including hyaluronan, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparin, heparan sulfate, and disaccharides, but not keratan sulfate or carrageenan. The 1,9-dimethylmethylene blue assay is used to detect sulfated GAG, but does not detect disaccharides, carrageenan, hyaluronan, or other unsulfated oligosaccharides.

For p ≤ 0.05, ** for p ≤ 0.01, *** for p ≤ 0.001.

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For p ≤ 0.05, ** for p ≤ 0.01, *** for p ≤ 0.001.
demonstrates a remarkable similarity in disaccharide composition upon carrageenan exposure and a major difference from the control untreated cells.

4. Discussion

The commonly used food additive carrageenan resembles the naturally occurring sulfated GAGs, since it is composed of repeating sulfated disaccharide units. However, the disaccharide linkages in carrageenan differ from those in the endogenous human GAGs, due to the presence of the unusual \( \alpha(3' \rightarrow 3)\beta\)-Gal bond. In contrast, chondroitin, dermatan, and keratan sulfates have \( \beta(1 \rightarrow 3) \) and \( \beta(1 \rightarrow 4) \) linkages and heparin and heparan sulfate have \( \beta(1 \rightarrow 4) \) and \( \alpha(1 \rightarrow 4) \) linkages between their sugar moieties. The \( \alpha(3' \rightarrow 3)\beta\)-Gal is recognized by anti-Gal antibodies [30] and appears to stimulate innate immune responses involving TLR4, BCL10, and NF-\( \kappa B \) [9–12]. Since carrageenan is so highly sulfated and resembles the endogenous GAG, the studies in this report were performed to determine the impact of carrageenan on activity of multiple sulfatase enzymes and the impact on cellular GAG composition.

Study findings indicate marked decline in activity of several sulfatase enzymes, including ARSB, ARSA, GALNS, STS, IDS, heparan sulfamidase, and SULF-1.2 following exposure to carrageenan. The assays were performed using standard methods that involve exogenous substrates and non-physiological conditions, and assays compared activity following carrageenan exposure vs. control under similar growth conditions. The assays have been developed to be sulfatase-specific and employ different temperature, pH, substrates, buffers, and inhibitors. Measurements consistently demonstrated that exposure of human colonic epithelial cells and mammary cells to carrageenan reduced the sulfatase activity. In contrast, cDNA microarray and cell-based ELISA did not demonstrate significant differences in sulfatase expression following carrageenan, suggesting a direct effect on enzyme function or on enzyme activation. The precise mechanism by which carrageenan inhibits enzyme activity is not yet determined, but may be attributable to an increase in sulfate, since sulfate is reported to inhibit enzyme activation. The precise mechanism by which carrageenan inhibits enzyme activity is not yet determined, but may be attributable to an increase in sulfate, since sulfate is reported to inhibit enzyme activation.

Since degradation of chondroitin–4-sulfate requires removal of the sulfate at the non-reducing end by ARSB (N-acetylgalactosamine-4-sulfatase), silencing ARSB activity by siRNA increased the abundance of cellular GAGs, in experiments reported in bronchial, mammary, renal, and colonic epithelial cells [16,27,33–35]. In association with the reduced ARSB activity, cellular sulfated GAG and CS were increased, secretion of vital molecules, including IL-8 and bradykinin, was reduced, and cellular sequestration of IL-8 and kininogen was increased, indicating the impact of sulfatase activity and GAG sulfation upon vital cell functions [33,35]. Silencing and overexpression of GALNS and ARSB produced corresponding changes in mRNA and protein expression of syndecan-1 and decorin in MCF-7 cells, demonstrating that changes in proteoglycans followed changes in GAG sulfation [27]. Also, since chondroitin-4-sulfation is critical for plasmoidal attachment, carrageenan, by effects on ARSB, and, thereby, on chondroitin sulfation, may have an impact on malarial infectivity [34].

In addition to the inhibitory effects on activity of sulfatase enzymes, and consistent with these effects, carrageenan exposure leads to marked increase in total cellular sulfated GAG content. This effect is attributable in part to impaired degradation of the GAGs when hydrolysis of sulfate groups is inhibited. Consistent with the inhibition of sulfatase enzyme activity and the overall increase in the GAG abundance, disaccharide analysis confirmed that the carrageenan exposure provokes profound changes in the composition of these cellular GAGs.

The CS/DS-derived disaccharides post-carrageenan included chondroitin sulfate D (\( \Delta\text{Di-dis} \)) and chondroitin sulfate E (\( \Delta\text{Di-dis} \)) which were not originally present. Galectin-3, -7, and -9 binding to sulfated GAGs has been linked to the extent of sulfation, suggesting that the effects of carrageenan on sulfatase activity may

---

### Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>∆Di-O5</th>
<th>∆Di-2S</th>
<th>∆Di-6S</th>
<th>∆Di-4S</th>
<th>∆Di-diS0</th>
<th>∆Di-diS9</th>
<th>∆Di-triS</th>
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</thead>
<tbody>
<tr>
<td>Untreated</td>
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<td>22.7</td>
<td>72.8</td>
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</tr>
<tr>
<td>Iota</td>
<td>8.1</td>
<td>20.1</td>
<td>71.2</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lambda</td>
<td>6.6</td>
<td>21.2</td>
<td>71.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

**Legend**: LC–MS = liquid chromatography-mass spectrometry. CS/DS = dermatan sulfate; DS = derman sulfate; ∆Di = disaccharide; ∆Di-O5 = ∆UA-GalNAc; ∆Di-4S = ∆UA-GalNAc4S; ∆Di-6S = ∆UA-GalNAc6S; ∆Di-2S = ∆UA2S-GalNAc; ∆Di-diS0 = ∆UA2S-GalNAc4S; ∆Di-diS9 = ∆UA2S-GalNAc6S; ∆Di-triS = ∆UA2S-GalNAc456S.

---

### Table 5

<table>
<thead>
<tr>
<th>Sample</th>
<th>∆Di-O5</th>
<th>∆Di-2S</th>
<th>∆Di-6S</th>
<th>∆Di-4S</th>
<th>∆Di-diS0</th>
<th>∆Di-diS9</th>
<th>∆Di-triS</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>6.9</td>
<td>-</td>
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<td>Lambda</td>
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<td>91.5</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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</tbody>
</table>

**Legend**: LC–MS = liquid chromatography-mass spectrometry. HS = heparan sulfate; HP = heparin; ∆Di = disaccharide; ∆Di-O5 = ∆UA-GalNAc; ∆Di-NS = ∆UA-GlCNs, ∆Di-6S = ∆UA-GalNAc6S; ∆Di-2S = ∆UA2S-GalNAc; ∆Di-2NS = ∆UA2S-GlCNs; ∆Di-NS6S = ∆UA-GlCN6S; ∆Di-triS = ∆UA2S-GlCN65S.

---

**Fig. 3.** HS/heparin disaccharide composition analysis by LC–MS. Extracted ion chromatography (EIC) of HS/HP disaccharide standards: (B), (C), (D) and (E) EIC of HS/HP disaccharides from the GAG samples CN, KCG, ICG, and LCG, respectively. [GAG = glycosaminoglycan; CS/DS = chondroitin sulfate/dermatan sulfate; KCG = kappa carrageenan; ICG = iota-carrageenan; LCG = lambda-carrageenan].
impact upon galectin binding [36]. Since galectins are linked to multiple cellular critical events, the carrageenan-induced changes in GAGs may lead to profound changes in cell functions and cell regulation through transcriptional effects, as well as altered GAG interactions [37–39]. Further analysis of the impact of carrageenan exposure may provide new insights into how GAG sulfation and sulfatases influence cell fate. The implications for human disease may be profound, since carrageenan is consumed in significant quantity in the human diet.

5. Conclusions

Exposure to the common food additive significantly reduced the activity of multiple sulfatase enzymes in human colonic and mammary epithelial cells. These changes in sulfatase activity were accompanied by marked increase in cellular GAGs. Disaccharide analysis demonstrated that CSD- and CSE-derived disaccharides were present following carrageenan exposure, but not in the untreated control. Analysis of HP/Hs-derived disaccharides demonstrated marked changes in the specific GAG composition following carrageenan, including the presence of ΔDi-6S, ΔDi-256S, and ΔDi-NS that were not originally present. These determinations indicate that exposure to the common food additive carrageenan has profound effects on sulfatase activity and GAG abundance and composition that may affect vital cell processes.

Acknowledgment

The authors acknowledge the contributions of Dr. Anuj Jain to performance of the cell-based ELISA experiments.

References