Published in final edited form as:

Biochim Biophys Acta. 2008 ; 1780(0): 973–982. doi:10.1016/j.bbagen.2008.03.019.

CARRAGEENAN-INDUCED NFκB ACTIVATION DEPENDS ON DISTINCT PATHWAYS MEDIATED BY REACTIVE OXYGEN SPECIES AND HSP27 OR BY BCL10

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Abstract

Carrageenans are highly sulfated polysaccharides that are widely used as food additives due to their ability to improve food texture. They are also widely recognized for their ability to induce inflammation in animal models of colitis. Recently, we reported that carrageenan (CGN) activated a pathway of innate immunity in human colonic epithelial cells mediated by Bcl10 (B-cell CLL/lymphoma 10). However, increases in phospho-IκBα and Interleukin-8 (IL-8) were not completely inhibited by silencing Bcl10, suggesting that CGN also influenced another mechanism, or mechanisms, of inflammation. In this report, we demonstrate that CGN increases production of reactive oxygen species (ROS) in human colonic epithelial cells. The combination of ROS quenching by the free radical scavenger Tempol and of Bcl10 silencing by siRNA completely inhibited the CGN-induced increases in nuclear NFκB (p65), phospho-IκBα, and secretion of IL-8. The CGN-induced increase in ROS was associated with declines in phosphorylation of MAPK 12 (p38γ), MAPK 13 (p38δ), and heat-shock protein (Hsp) 27. The CGN-induced decline in phospho-Hsp27 was reversed by co-administration of Tempol (100nM), but unaffected by silencing Bcl10. Since Hsp27 phosphorylation is inversely associated with phosphorylation of the IκBα kinase (IKK) signalosome, CGN exposure appears to affect the IKK signalosome by both the catalytic component, mediated by ROS-phospho-Hsp27, and the regulatory component, mediated by Bcl10 interaction with IKKγ (Nemo). Hence, the CGN-activated inflammatory cascades related to innate immunity and to generation of ROS may be integrated at the level of the IKK signalosome.

Keywords

Carrageenan; Reactive Oxygen Species (ROS); Bcl10 (B-cell CLL/lymphoma 10); Hsp27; MAPK; NFκB; IL-8; Colonic Epithelial Cells; sulfate assimilation pathway

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DISCLOSURES: None

1. Introduction

Carrageenans (CGN) are highly sulfated polysaccharides derived from red seaweeds (*Rhodophyceae*), predominantly of the genera *Chondrus*, *Eucheuma*, *Gigartina*, and *Iridaea*. Structurally, CGN resembles the naturally occurring sulfated glycosaminoglycans (GAG), since CGN is a polymer composed of sulfated disaccharide units with α -1,3 and β -1,4 galactosidic linkages. The biological reactivity of CGN may be attributable to some extent to its activation of a pathway of innate immunity, due to CGN's unique structure that includes the immunogenic gal(α -1,3)gal epitope [1,2]. Of mammals, only humans and Old World apes lack the alpha-1,3-galactosyl transferase enzyme that is required to form the alpha-1,3-galactosidic linkage, recognized by the anti-Gal antibody [3,4].

Previously, we reported the unexpected finding that CGN activates an innate immune pathway of inflammation, mediated by Bcl10 in human colonic epithelial cells (CEC) [5]. Activation of this cascade induced the nuclear translocation of NF κ B (p65) and enhanced IL-8 secretion in NCM460 cells and normal human colonocytes in tissue culture. Recently, we identified Toll-like receptor 4 (TLR4) as the cell surface receptor for this innate immune pathway that is stimulated by CGN and mediated by Bcl10 [6].

The predominant mechanism of CGN-induced increases in NF κ B and IL-8 is Bcl10-mediated, since Bcl10 silencing by small interfering siRNA produces marked declines in CGN-induced increases in IL-8 and phospho-I κ B [5]. However, the effects of CGN on production of inflammation in colonic epithelial cells are not completely explained by this pathway. In this report, we present the effects of CGN on production of ROS and of redox-associated enzymes, and the effects of free radical scavenging on the CGN-induced inflammatory cascade, in order to more completely explain the mechanisms by which CGN exposure triggers an inflammatory response in human intestinal epithelial cells. Since CGN is one of the most highly sulfated molecules known, we hypothesize that some of the biological effects of CGN may be attributable to its abundant sulfate groups, and that the increased generation of ROS and of the responsive redox enzymes comprise components of a sulfate assimilation pathway in the intestinal cells. We note that the exposure of the human colon to CGN from the average Western diet is anticipated to be greater than the exposure to colonic epithelial cells in our experiments, so these pathways may contribute to harmful effects of CGN on the human intestine *in vivo*.

2. Materials and Methods

2.1. Cell culture of colonocytes

The NCM460 cell line is a human colonic epithelial cell line that was originally derived from the normal mucosa of a 68-year-old Hispanic male [7]. Cells were grown in M3:10TM media (INCELL, San Antonio, TX) and maintained at 37°C in a humidified, 5% CO₂ environment with media renewal at two-day intervals. Confluent cells in T-25 flasks were harvested by EDTA-trypsin and sub-cultured in multi-well tissue culture plates. In the majority of experiments, cells were treated with λ CGN (1 μ g/ml) for 24 hours. Spent media were collected from control and treated wells at specified intervals and stored at -80°C pending analysis. Cells were harvested by scraping, and total cell protein was measured by BCATM Protein Assay Kit (Pierce, Rockford, IL), using bovine serum albumin as a standard.

De-identified colon specimens were obtained at the time of colectomy through an established protocol approved by the Institutional Review Board of the University of Illinois at Chicago (UIC). Normal tissue that was several centimeters away from any macroscopic lesion was maintained under sterile conditions, and small tissue samples were obtained by scraping from the underlying muscle. Colonocytes were dispersed from the scraped tissue by

digestion with Collagenase IV (Worthington Biochemical Corp, Lakewood, NJ) for 6 hours in a Tissue Digestion Mixture, as previously described [5]. Dispersed cells were collected after successive centrifugations and plated in M3:10™ media (INCELL, San Antonio, TX), and then maintained at 37°C in a humidified, 5% CO₂ environment. Lambda (λ), iota (ι), and kappa (κ) carrageenans (CGN) were obtained commercially (Sigma, St. Louis, MO), and dissolved in sterile water at room temperature (λ) or 37°C.

2.2. Free radical scavengers Tempol and Tiron

Tempol (2,2,6,6-tetramethylpiperidinyloxy, Axxora Life Sciences, Inc., San Diego, CA) and Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid; Acros Organics, Thermo-Fisher, New Jersey) are free radical scavengers able to detect intracellular oxygen radicals. Tempol and Tiron were used at concentrations of 100 nM, and the human colonic epithelial cells (CEC) were treated with either Tempol or Tiron in the presence of λCGN for 1-24 hours.

2.3. Measurement of reactive oxygen species (ROS)

The production of reactive oxygen species (ROS) by λCGN was measured by the method of Ndengele *et al* [8]. Cells were harvested from T-75 flasks and seeded in a 96-well tissue culture plate. After 24 hours, the media were changed, and treatment with CGN and the free radical scavenger Tempol or Tiron was started. At the end of the treatment, the medium in each well was removed, and cells were washed with Hank's Balanced Salt Solution (HBSS). Cells were incubated at 37°C for 60 minutes with 200 μl of HBSS containing 10 μM hydroethidine (HE), which detects intracellular superoxide anion by changing from blue to red fluorescence in the presence of O₂⁻ when the oxyethidium derivative forms. Medium was removed, and fresh HBSS (200 μL/well) added. Intracellular HE fluorescence emitted by the cells was measured using a microplate fluorescence reader (FL600, Bio-Tek Instruments Inc.) at 488 nm excitation with a 610-nm emission filter.

2.4. Measurement of IL-8 secretion by ELISA

Secretion of IL-8 in the spent media of control and λCGN-treated NCM460 and primary colonic cells was measured using the DuoSet ELISA kit for human IL-8 (R&D Systems Inc, Minneapolis, MN), as described previously [5,9]. Sample values were normalized with total protein content (BCA™ protein assay; Pierce) and expressed as pg/mg protein.

2.5. Measurement of inducible nitric oxide synthase (iNOS) by ELISA

To evaluate the possible induction of iNOS activity, NCM460 cells were treated with λCGN (1 μg/ml) for 1, 4 and 24 hours. Cells were harvested, and cell lysates prepared from control and treated wells in a lysate buffer provided with the human iNOS ELISA kit (R&D Systems). The iNOS in the samples was captured in the microplate wells that were coated with specific iNOS monoclonal antibody. The captured iNOS was detected by a second monoclonal antibody to iNOS conjugated with horseradish peroxidase (HRP). The bound enzyme activity was detected by developing the color with hydrogen peroxide/tetramethylbenzidine (TMB) chromogenic substrate, and the intensity of color was measured at 450 nm with a reference filter of 570 nm in an ELISA plate reader (SLT, Spectra). The iNOS units in the samples were extrapolated from a standard curve plotted using known concentrations of iNOS standards. The sample values were normalized with total protein content (BCA protein assay, Pierce) and expressed as U/mg protein, and 1 U/ml of partially purified human iNOS approximately equals 1 ng/ml iNOS.

2.6. Measurement of Superoxide Dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured in the NCM460 cells before and after treatment with CGN (1 μg/ml for 1 and 24 hours), and with or without coincident exposure

to Tempol, by SOD enzyme assay kit (Cayman Chemical, MI). The assay utilized a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. SOD modifies the superoxide radicals, so when more SOD is present in the test sample, less superoxide is present to interact with the tetrazolium salt, and the color that develops is less intense. Cells from control and treated wells were harvested, washed in phosphate-buffered saline (PBS), and sonicated in cold 20 mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210mM mannitol and 70 mM sucrose. The sonicates were centrifuged at $1500 \times g$ for 5 minutes, and supernatants collected. 10 μ l of SOD standards or samples were combined with 200 μ l of radical detector. The reaction was initiated by 20 μ l of xanthine oxidase, and preparations were incubated at room temperature for 20 minutes. The intensity of color was measured at 450nm in ELISA Plate reader (SLT, Spectra), and intensity of color was inversely proportional to SOD content. The SOD concentrations were extrapolated from a standard curve plotted using known concentrations of SOD standards.

2.7. Silencing Bcl10 mRNA Expression by siRNA

Small interfering RNA (siRNA) for Bcl10 was obtained commercially (Qiagen, Valencia, CA). NCM460 cells were grown to 60-70% confluency in 12- well tissue culture plates. The medium of the cells was aspirated and replaced with 1.1 ml of fresh growth medium with serum. 75 ng/well of Bcl10 siRNA (Qiagen Inc. Valencia, CA), was mixed with 100 μ l/well of serum free medium and 6 μ l/well of HiPerfect Transfection Reagent (Qiagen). The mixture was incubated for 10 minutes at room temperature to allow the formation of transfection complexes, and then added dropwise onto the cells. The culture plate was swirled gently, and the treated cells and controls were incubated at 37°C in a humidified, 5% CO₂ environment. After 24 hours, the medium was replaced by fresh growth medium. Silencing of Bcl10 expression was demonstrated by absence of the Bcl10 band in Western blot of the NCM460 cell lysate when probed with a mouse monoclonal Bcl10 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) [5].

2.8. Proteome Profiler Array

The NCM460 cells in culture were treated with 1 μ g/ml of λ CGN for 24 hours. Cell lysates from control and treated cells were prepared in lysis buffer provided with the proteome profiler (R&D), which is designed to detect 21 phosphorylated kinases or related signaling mediators in duplicate simultaneously. Samples were incubated with the nitrocellulose membrane array overnight at 4° C. After washing off the unbound cell lysates, the array was incubated with a cocktail of phospho-site specific biotinylated antibodies for 2 hours at room temperature, and then with Streptavidin-HRP for 30 minutes. Signals were developed with chemiluminescent reagents, and recorded on X-ray film. Densities of individual dots corresponding to a phosphorylated kinase were measured by Image J software (NIH) and compared between treated and control samples.

2.9. Measurement of total and phosphorylated Hsp27 by ELISA

Total and Phospho-Hsp27 in the control and treated cells were measured by a FACE-ELISA (fast-activated cell-based ELISA; Active Motif, CA). NCM460 cells were grown in 96-well plates and treated with λ CGN (1 μ g/ml $\times$ 24 hours). Following CGN exposure, the cells were washed and fixed with 4% formaldehyde in PBS. After 20 minutes, the formaldehyde was aspirated, cells were washed with PBS, and then treated with quenching buffer, to suppress endogenous peroxidase activity. Next, cells were treated with blocking buffer and incubated overnight at 4°C with specific monoclonal antibodies against either total Hsp27 or phospho-Hsp27. Binding of primary antibodies to cells was detected by secondary antibody-HRP, and bound HRP activity was developed by hydrogen peroxide/TMB substrate. The reaction was stopped by 2N sulfuric acid, and the optical density was measured in an ELISA plate reader (SLT, Spectra) at 450nm. After the reading, cells were washed thoroughly and

incubated in crystal violet solution for 30 minutes, followed by 1% SDS (sodium dodecyl sulfate) solution for one hour. Color was proportionate to the cell number and was measured by optical density using an ELISA plate reader at 592 nm. This reading was used to normalize the first reading (either total or phospho-Hsp27 per equal number of cells), and the result was expressed as % of control for the total Hsp27 or the phospho-Hsp27.

2.10. Measurement of Cox-2 activity

Cyclo-oxygenase-2 level in CGN-treated NCM460 cells was determined by Cox-2 ELISA (Calbiochem, CA). Cells were grown 6-well tissue culture plates, treated with λ CGN ($1\mu\text{g}/\text{ml} \times 24$ hours), harvested by scraping, and cell lysates were prepared from control and treated wells. Cox-2 in the lysates was determined by solid-phase ELISA in which immobilized Cox-2 molecules were measured by detector antibody and conjugated secondary antibody complex. Color was developed by adding hydrogen peroxide/TMB mixture, and intensity of the color was read at 450nm in an ELISA plate reader (SLT, Spectra).

2.11. Measurement of Prostaglandin 2E by ELISA

Prostaglandin E2 (PGE2) was detected by ELISA (R&D) using a competitive colorimetric assay in which the color development was inversely proportional to the PGE2 content of the cell culture supernatant. NCM460 cells were exposed to λ CGN ($1\mu\text{g}/\text{ml} \times 24$ hours), and the spent media collected and added to the wells of a 96-well microplate that were coated with a goat anti-mouse antibody. A fixed amount of HRP-labeled PGE2 competed with the supernatant PGE2 in binding to a mouse monoclonal PGE2 antibody that was added to the wells. During incubation, the PGE2-mouse monoclonal antibody complex bound to the coated wells. Following washes, hydrogen peroxide/TMB was added to develop color with the HRP-labeled PGE2, and the color was inversely proportional to the PGE2 content of the supernatant media that competed for the binding sites. PGE2 concentration was determined by comparison to a standard curve prepared with known concentrations of PGE2.

2.12. Measurement of nuclear NF κ B by ELISA

Nuclear extracts were prepared from treated and control NCM460 cells using a nuclear extraction kit (Active Motif). Activated NF κ B in the samples was determined by oligonucleotide-based ELISA (Active Motif). Treated and control samples were added and incubated in 96-well microtiter wells, and the activated NF κ B in the nuclear extracts attached to a consensus nucleotide sequence (5'-GGGACTTCC-3') that was coated onto the microtiter wells. Attached NF κ B was captured by antibody to NF κ B (p65) and detected by an anti-rabbit-HRP-conjugated IgG. Color development was performed with hydrogen peroxide/TMB chromogenic substrate, and intensity of the developed color proportionately represented the quantity of NF κ B in each sample. The specificity of the binding of NF κ B of the samples with the nucleotide sequence was determined by comparison to the control binding, determined by adding either free consensus nucleotide or mutated nucleotide to the reaction buffer. The sample values were normalized with the total cell protein determined by protein assay kit (Pierce).

2.13. Measurement of Phospho-I κ B α (Serine32) by ELISA

Phospho-I κ B α was determined in the NCM460 cells following exposure to Tempol and to Bcl10 siRNA by an ELISA (Cell Signaling) that detects phosphorylation of Serine 32. A mouse monoclonal antibody against I κ B α was coated onto the microwells of a 96-well plate into which lysates of NCM460 cells were added. The NCM460 cells had been treated with λ CGN ($1\mu\text{g}/\text{ml} \times 24$ hours), Tempol ($100\text{ nM} \times 24$ hours), Bcl10 siRNA or control siRNA ($\times 24$ hours, as described above), as well as combinations of these exposures.

Phospho-I κ B α in the cell lysate was captured by the antibody, the wells were extensively washed, and a second antibody added to detect the captured phospho(Ser 32)-I κ B α protein. An HRP linked anti-rabbit antibody was used to recognize the bound detection antibody, and hydrogen peroxide/TMB was added for color development. The optical density is proportional to the quantity of phospho-I κ B α protein present.

2.14. cDNA microarray

cDNA microarray was performed in order to assess the extent of cellular changes induced by CGN exposure. The Affymetrix U133 plus 2.0 system was used, following extraction of total RNA by RNeasy minikit (Qiagen) [10]. RNA was extracted from replicate biological samples of treated and control samples and separate chips were prepared. Each chip had 11 probeset pairs (22 probes total) for every gene identified. Data from the probesets were normalized and combined. Signal intensities were normalized using background correction, mismatch correction, and quantile normalization. Pair-wise comparisons of control vs. treated mean values were used to compare the samples. 526 genes had fold changes of 1.5 or greater between control and treated. Of these, 184 genes were differentially expressed with a Bonferroni corrected p-value of ≤ 0.05 [11]. A total of 790 transcripts had p-values less than 0.001 prior to correction out of the 54,613 transcripts present on the Human U133 2.0 Gene Chip.

2.15. Quantitative RT-PCR of sulfiredoxin

Quantitative analysis of changes in gene expression was performed using the Stratagene Mx3000P system. Total cellular RNA was prepared using the RNeasy Mini Kit (Qiagen). Quantitative reverse transcription polymerase chain reaction (QRT-PCR) was performed using the 1-step Master Mix (Stratagene Brilliant® for QRT-PCR). Primers were selected using Primer3 software [12], and forward and reverse primers were prepared commercially (Integrated DNA Technologies). Primers for Sulfiredoxin (NM_080725) were: F: 5'-TTC CCC CTT TCT CTC TGT CTC-3' and R: 5'-GCC TTA CTC TGT TCC CTT TCC-3'.

Cycle threshold (Ct) for the genes of interest in CGN-treated and untreated samples was compared to cycle threshold for β -actin. Fold-changes in expression were determined from the differences in Ct using the formulae:

$$\begin{aligned}\text{Fold Change} &= 2^{\Delta 3} \quad \text{with} \quad \Delta 3 \\ &= \Delta 1 - \Delta 2; \Delta 1 \\ &= C_{t\text{control target gene}} - C_{t\text{control actin}} \quad \text{and} \quad \Delta 2 \\ &= C_{t\text{treated target gene}} - C_{t\text{treated actin}}.\end{aligned}$$

2.16. Okadaic acid inhibition of phosphatase activity

Okadaic acid (OA) inhibits phosphatase activity, with inhibition of Protein Phosphatase 2A (PP2A) greater than inhibition of Protein Phosphatase 1 (PP1). To determine the effect of OA on CGN-induced activation, NCM460 cells were co-incubated with OA 5nM and CGN 1 μ g/ml $\times$ 24 hours at 37°C in 5%CO₂. IL-8 secretion was measured by ELISA, as described above.

2. 17. Statistical analysis

Data are the mean $\pm$ Standard Deviation (SD) of three biological replicates and two technical replicates of each, unless stated otherwise. Statistical significance was determined either by paired, two-tailed t-test or by one-way ANOVA followed by a post-hoc Tukey-

Kramer test for multiple comparisons using Prism or InStat software. *** represents $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

3. Results

3.1. CGN-induced increase in reactive oxygen species (ROS)

Production of reactive oxygen species (ROS) in NCM460 cells and in primary human colonic epithelial cells (CEC) was markedly increased, following exposure to λ CGN (1 μ g/ml) at 1, 4, and 24 hours (**Figures 1A and 1B**). When treated with CGN, the ROS production in the primary CEC increased to 5 times the control within the first hour of treatment, peaked at eight times the baseline within 4 hours, and remained at this level at 24 hours. Differences between CGN-exposed and controls are statistically significant at all time points ($p < 0.001$, 1-way ANOVA and Tukey's post-test; $n = 3$).

3.2 Free radical scavengers reduce CGN-associated increases in ROS and IL-8

When NCM460 or primary human CEC were treated with CGN in the presence of the free radical scavenger Tempol, significant reductions in production of ROS occurred (**Figures 2A and 2B**) ($p < 0.001$, 1-way ANOVA and Tukey's post-test; $n = 3$). In the presence of 100nM of Tempol at 1, 4, and 24 hours, ROS levels declined to less than baseline values.

CGN-induced increases in IL-8 in the NCM460 and primary human CEC declined significantly in the presence of the free radical scavengers Tempol and Tiron (**Figures 3A, 3B, 3C**; $p < 0.001$, 1-way ANOVA and Tukey's post-test; $n = 3$). In the presence of Tempol, IL-8 declined from CGN-induced peak of 1238.2 (± 46.1) pg/mg protein to 835.9 (± 29.7) pg/mg protein, from a control value of 615.5 (± 8.0) pg/mg protein at 24 hours, suggesting that ~65% of the CGN-associated increase in IL-8 was attributable to an effect of ROS. However, baseline IL-8 values also declined in the presence of Tempol, indicating that less than this percentage of the CGN-induced increase was attributable to ROS effects.

3.3. Increased Superoxide Dismutase (SOD) activity following CGN

When NCM460 cells were treated with CGN (1 μ g/ml $\times$ 1 hr and 24 hours), SOD activity doubled within 1 hour, and remained increased at 24 hours (**Figure 4**). The differences in SOD activity at 1 hour and at 24 hours between untreated control and CGN-treated cells and between CGN-treated and CGN+Tempol-treated cells were statistically significant ($p < 0.001$ level, 1-way ANOVA and Tukey's post-test; $n = 3$).

3.4. CGN-associated increases in redox enzymes

Increase in cyclo-oxygenase 2 (Cox-2) (**Figure 5A**) occurred in the NCM460 cells, following exposure to CGN (1 μ g/ml $\times$ 24 hours). Cox-2 level increased to 4-fold times the control, from 2.79 (± 0.08) ng/mg protein to 11.64 (± 0.39) ng/mg protein ($p < 0.0001$, unpaired t-test, two tail; $n = 3$). Consistent with the increase in Cox-2, prostaglandin E2 (PGE2) increased over six-fold, from 63.4 (± 4.0) pg/mg protein to 388.2 (± 20.8) pg/mg protein, following exposure to CGN ($p < 0.0001$, unpaired t-test, two tail; $n = 3$). In contrast, no increase in iNOS concentration was found following exposure to λ CGN for 1, 4, and 24 hours (**Figure 5B**) ($n = 6$). iNOS levels were very low in the control cells and did not increase following CGN exposure.

cDNA microarray was performed in NCM460 cells following exposure to λ CGN 1 μ g/ml $\times$ 4 days. Results demonstrated highly significant increases in the expression of glutathione synthetase (also known as glutamate cysteine ligase) and selenoprotein-S, and marked decline in NADPH oxidase (NOX1) (**Table 1**). Expression of the redox enzymes sulfiredoxin and thioredoxin reductase 1 was also increased to 1.3-fold times the control

value in the cDNA microarray, but the increase did not achieve statistical significance, when corrected for multiple comparisons (uncorrected $p=0.00028$ for thioredoxin reductase 1). QRT-PCR for the enzyme sulfiredoxin confirmed an increase in expression and was statistically significant ($p=0.002$, unpaired t-test, two-tail, $n=4$).

3.5. CGN exposure inhibits phosphorylation of Hsp27, MAPK 12, and MAPK 13

NCM460 cells were treated with CGN ($1 \mu\text{g/ml} \times 24$ hours), and control and treated cell lysates were tested using the Proteome Profiler Array (**Figure 6A**). Densitometric analysis (**Figure 6B**) confirmed the marked differences between control and following CGN. Notably, CGN exposure reduced the intensity of phospho-Hsp27, phospho-MAPK12, (p38 γ) and phospho-MAPK13 (p38 δ), as measured by densitometry (**Figure 6C**) ($p<0.001$, 1-way ANOVA and Tukey's post-test, $n=3$). Since the phosphorylation of Hsp27 is inverse to the phosphorylation of IKK α/β , these CGN-induced declines in phospho-Hsp27 can affect the phosphorylation of I κ B α [13-15].

3.6. CGN-induced decline in Phospho-Hsp27 is attributable to ROS production

Total Hsp27 and phospho-Hsp27 were measured by ELISA in control and CGN-treated NCM460 cells. Total Hsp27 (**Figure 7A**) did not change following CGN exposure, in contrast to a significant decline in phospho-Hsp27 (**Figure 7B**). Combined exposure to CGN and Tempol produced no change in the total Hsp27, but reversed the CGN-induced decline in phospho-Hsp27. Tempol alone increased the baseline phospho-Hsp27 by $24.3 (\pm 4.6) \%$. CGN alone reduced the phospho-Hsp27 by $\sim 40\%$, but in combination with Tempol increased the value by $28.5 (\pm 9.5) \%$ over the baseline. These results demonstrate that the CGN-induced increase in ROS production was responsible for the decline in phospho-Hsp27, since it was reversed by the addition of an ROS scavenger. Differences from control following Tempol and CGN are statistically significant ($p<0.001$, 1-way ANOVA followed by Tukey-Kramer post-test, $n=3$).

3.7. Okadaic acid inhibits the CGN-induced increase in IL-8

Combined incubation of the NCM460 cells with okadaic acid 5 nM and λ CGN $1 \mu\text{g/ml} \times 24$ hours resulted in marked reduction in the CGN-induced increase in IL-8 ($p<0.001$, 1-way ANOVA followed by Tukey-Kramer post-test, $n=3$) from $1243.4 (\pm 79.0) \text{ pg/mg protein}$ to $865.4 (\pm 34.0) \text{ pg./mg protein}$ (**Figure 8**). This decline, and the decline in the baseline IL-8 secretion, suggest that PP2A activity is required for CGN-induced activation of IL-8, as well as for the baseline level of IL-8 secretion.

3.8. CGN-induced decline in phospho-Hsp27 is independent of Bcl10

Bcl10 knockdown by siRNA had no effect on total Hsp27 (**Figure 9A**) or phospho-Hsp27 (**Figure 9B**), consistent with independent pathways of CGN-induced activation through Bcl10 and through ROS.

3.8. CGN-induced increases in activation of NF κ B and phospho-I κ B α and in secretion of IL-8 depend on both Bcl10 and ROS

When NCM460 cells were treated with siRNA for Bcl10, Tempol, and λ CGN, in combination with appropriate controls, the nuclear NF κ B content varied (**Figure 10A**). Both Tempol and Bcl10 siRNA were required to inhibit the CGN-induced increase in nuclear NF κ B. Transfection of control cells with only Bcl10 siRNA had no effect on the activation of NF κ B, whereas Tempol alone reduced the activated NF κ B level $\sim 25\%$ from the baseline level. CGN exposure increased the nuclear NF κ B by 100% over the baseline level; Bcl10 silencing decreased the CGN-induced activation by $\sim 67\%$ and Tempol reduced it by $\sim 56\%$.

Bcl10 silencing and Tempol in combination totally inhibited the CGN-induced activation of NF κ B.

Similarly, measurements of phospho-I κ B α (Serine 32) by ELISA demonstrated that its CGN-induced increase was attributable to both Bcl10 and to ROS. Decline in phospho-I κ B α to less than baseline required both siRNA for Bcl10 and Tempol, a quencher of ROS (**Figure 10B**).

When the IL-8 concentrations in the spent media were measured (**Figure 10C**), it was evident that both Bcl10 siRNA and Tempol were required to eliminate the CGN-induced increase in IL-8. Tempol reduced the CGN-induced increase in IL-8 less than Bcl10 silencing did, and some of the Tempol-associated decline was attributable to its effect on the baseline IL-8 secretion. These findings demonstrate that CGN-induced IL-8 secretion is mediated predominantly through the Bcl10-mediated pathway, with a secondary effect through ROS. The CGN-induced pathways are depicted schematically in **Figure 11**.

DISCUSSION

Bcl10 is well-recognized as a mediator of a mechanism of inflammation in immune cells [16-19]. Recent reports, including our work in human colonic epithelial cells, have also established a role for Bcl10 in an inflammatory pathway in non-immune cells, including mouse embryonic kidney cells and hepatocytes [5,8,20-22]. Bcl10 is recognized as a mediator of constitutive activation of NF κ B in the MALT lymphomas, in which there is a translocation involving Bcl10 [23-25]. The Bcl10 effects appear to be mediated through its interaction with IKK γ (or Nemo), the regulatory component of the IKK signalosome. Bcl10 has been shown to co-localize with Nemo, and the modifications of Nemo by phosphorylation and ubiquitination are critical for the phosphorylation and ubiquitination of I κ B α [26,27]. The phosphorylation of I κ B α following CGN, which we have reported previously [5], enables the nuclear translocation of NF κ B and the enhanced secretion of IL-8 by the effect of NF κ B on the IL-8 promoter. In the experiments presented in this report, Bcl10 silencing had no effect on the phosphorylation of Hsp27. In contrast, phospho-Hsp27 was increased by Tempol and inhibited by CGN. Hence, Bcl10 does not influence the nuclear translocation of NF κ B or the secretion of IL-8 by an ROS-phospho-Hsp27-mediated mechanism.

The phosphorylations of the p38 MAPK kinases and Hsp27 and of the IKK signalosome complex have been reported to involve PP2A [28-31]. The phosphorylation of Hsp27 has been reported to enhance association with IKK β and result in reduced IKK activity. Since decline in phosphorylation of the IKK complex produces reduction in phosphorylation of I κ B α and reduced NF κ B nuclear translocation, these effects are consistent with a role for phospho-Hsp27 as an inhibitor of the stress response [29,30]. The demonstrations that CGN induces decline in phospho-Hsp27 and that okadaic acid treatment reduces the CGN-associated increase in IL-8 are consistent with the increases in nuclear NF κ B, and in phospho-I κ B α and IL-8 reported previously [5]. CGN exposure modifies both the phosphorylation of the catalytic components of the IKK signalosome through the phospho-Hsp27-mediated response and the regulatory Nemo domain, through the Bcl10 innate immune response. These combined effects indicate that CGN influences the nuclear translocation of NF κ B by a complex series of interactions, involving both Bcl10 and ROS-mediated pathways.

CGN exposure generates multiple signals, related to ROS, innate immunity, and modification of phosphorylation cascades. The unique chemistry of CGN, including its resemblance to the naturally occurring glycosaminoglycans, its high degree of sulfation, and

its unusual alpha-1,3-galactosidic linkage, presents three prongs by which it may interfere with intrinsic biological processes. We have previously reported that CGN exposure is associated with cell cycle arrest and with activation of mechanisms related to polyp formation involving BMP-wnt- β -catenin, further demonstrations of its marked biological reactivity [11,32].

The increases in sulfiredoxin and glutathione synthetase expression following exposure to CGN suggest a possible upregulation of mechanisms related to metabolism of sulfate, since λ CGN is so highly sulfated. Activation of glutamate cysteine ligase (glutathione synthetase), the first step in the cellular response to oxidative stress [33-35], and of sulfiredoxin, which functions as a high-end salvage pathway that follows periredoxin [36-38], suggests that the effects of CGN on oxidative stress are profound and extensive. Although oxidative stress is generally recognized in relation to exposure to oxy- and nitroso-groups, the induction of glutathione synthetase and of sulfiredoxin suggests the activation of sulfate-specific redox mechanisms following exposure to a sulfate load. We hypothesize that CGN-associated sulfate groups become part of the human intestinal epithelial cell's electron acceptor pool and disrupt the normal redox balance. The sulfate assimilation pathway in plants, yeast, and protists provides a prototype for a series of reactions that reduce sulfate groups and metabolize sulfate so that it can be re-utilized in sulfotransferase reactions [39-45].

Exposure to the highly sulfated CGN generates marked increase in reactive oxygen species in the intestinal cells, leading to protective cellular responses to contain the effects of the increased ROS. In mammalian cells, sulfur dioxide and Na_2SO_3 have been associated with marked changes in ROS and activation of redox pathways [46-49]. In addition to the significant increases in glutathione synthetase and sulfiredoxin, superoxide dismutase and selenoprotein-S increased and NADPH oxidase declined significantly. Also, CGN treatment provoked significant increase in COX-2, consistent with prior reports of elevations of cyclooxygenase in intestinal inflammation [50-52]. In contrast, no effect on i-NOS was observed, although increased i-NOS was detected following CGN exposure in other experimental models [53-55] and in the inflamed intestine [56,57]. The increased production of i-NOS in other studies may be attributable to the inclusion of other cell types in the tissue samples from inflammatory exudates.

We have shown that CGN exposure to human colonic epithelial cells in tissue culture induced increases in NF κ B nuclear translocation and IL-8 secretion through two distinct pathways, mediated by Bcl10 and ROS. The Bcl10-mediated pathway involves the innate immune response and is responsible for the majority of the effects of CGN in the inflammatory cascade leading to IL-8. The ROS-mediated pathway, that is associated with decline in phospho-Hsp27, impacts on the phosphorylation of the IKK signalosome, thereby mediating the nuclear translocation of NF κ B. Sulfate-related responses suggest activation of a sulfate assimilation pathway in the human intestinal cells following exposure to the highly sulfated CGN. The study findings indicate that CGN significantly modifies vital processes in human colonic epithelial cells, and intensify concern about the appropriateness of the ongoing inclusion of CGN as an additive in food for human consumption.

Acknowledgments

GRANTS: This work was funded in part by Department of Veterans Affairs and by NIDDK (DK54016 and DK68324) to PKD.

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Figure 1. Increased ROS production following CGN exposure in IEC

NCM460 cells (**1A**) and normal primary colonocytes (**1B**) in culture were treated with CGN (1 μ g/ml) for 1, 4 and 24 hours and reactive oxygen species (ROS) production was measured by a fluorometric assay using 10 μ M hydroethidine (HE) as substrate. CGN stimulated ROS production within 1 hour; 4 and 24 hour values were similar, with levels 8-9 times the control. Differences between the control and treated cells at the same time point are statistically significant ($p < 0.001$, 1-way ANOVA with Tukey's post-test, $n = 3$).

A. **B.**

Figure 2. CGN-induced increase in ROS secretion is inhibited by ROS scavenger Tempol in IEC When NCM460 cells (**2A**) or normal primary colonocytes (**2B**) were treated with CGN (1 μ g/ml) for 24 hours in combination with 100nm Tempol), the production of ROS was markedly diminished, to values less than baseline ($p < 0.001$, 1-way ANOVA with Tukey's post-test, $n=3$).

B.

C.

Figure 3. Stimulation of IL-8 secretion from NCM460 cells by CGN inhibited by ROS scavengers NCM460 cells in culture were treated with CGN (1 μ g/ml) for 1, 4 and 24 hours in presence or absence of 100 nM Tempol (3A) or for 24 hours in the presence or absence of 100 nM Tiron (3B), and secretion of IL-8 in the spent medium was assayed by IL-8 ELISA. CGN increased IL-8 secretion ~100%, but in the presence of Tempol this increase declined ~65%. Similarly, when normal human colonocytes were exposed to CGN (1 μ g/ml $\times$ 24 hours) with or without Tempol (3C), marked changes in the IL-8 secretion occurred. Increases with CGN exposure and declines with ROS scavengers are statistically significant ($p < 0.01$ for comparison between CGN-treated and CGN+Tempol at 1 hour; all other comparisons $p < 0.001$, 1-way ANOVA with Tukey's post-test, $n=3$).

Figure 4. Increased SOD Activity in CGN-treated Cells

CGN exposure markedly increased the SOD activity in the NCM460 cells. Co-administration of Tempol with CGN inhibited the increase in SOD. Differences between CGN-treated vs. control and CGN-treated vs. CGN+Tempol-treated were statistically significant at both 1 and 24 hours ($p < 0.001$, 1-way ANOVA with Tukey's post-test, $n=3$).

Figure 5. Increase in Cox-2, but not i-NOS, following exposure to CGN

CGN exposure increased the Cox-2 production in the NCM460 cells, to ~400% baseline, from $2.79(\pm 0.08)$ ng/mg protein to $11.64 (\pm 0.39)$ ng/mg protein (**5A**). The observed difference between the control and treated groups was statistically significant ($p < 0.0001$, unpaired t-test, two-tail, $n=3$). Prostaglandin E2 was measured by ELISA in the NCM460 cells following exposure to λCGN ($1 \mu\text{g/ml} \times 24$ hours) (**5B**). Six-fold increase occurred from $63.4(\pm 4.0)$ pg/mg protein to $388.2 (\pm 20.8)$ pg/mg protein ($p < 0.0001$, unpaired t-test, two-tail, $n=3$). iNOS was measured in the control and CGN-treated cells, and showed no change following exposure to CGN for 1 and 24 hours ($n=6$) (**5C**). 1U/ml of partially purified human iNOS approximately equals 1ng/ml iNOS.

Figure 6. Decreases in Phosphorylated MAPK12, MAPK13 and Phospho-Hsp27 following CGN exposure

NCM460 cells in culture were treated with CGN for 24 hours, and the cell content of 21 phospho-proteins was analyzed by the Phospho-Proteome Profiler. Densitometry was performed of the control (**6A**) and CGN-treated (**6B**) arrays, and statistical significance of the differences in intensity determined. Reduced intensity of the replicate spots for MAPK12 (p38 γ), MAPK 13 (p38 δ), and Hsp27 is evident and differences in intensity are statistically significant ($p < 0.001$, 1-way ANOVA with Tukey's post-test). Densitometric analysis (**6C**) confirms the change in intensity of phosphorylated-MAPK 12, MAPK13, and Hsp27 following CGN exposure ($p < 0.001$, 1-way ANOVA with Tukey's post-test, $n=3$).

A. **B.**

Figure 7. CGN reduces and Tempol increases phospho-Hsp27

NCM460 cells in culture were treated with CGN and Tempol, and total and phospho-Hsp27 were measured by ELISA. Total Hsp27 (**7A**) was not changed following CGN or Tempol, but Tempol increased the phospho-Hsp27 (**7B**) by 24.3% (± 4.6) and CGN reduced the phospho-Hsp27 to 60.9% (± 4.2) of baseline. These observed differences were significant ($p < 0.001$, 1-way Anova with Tukey's post-test, $n=3$).

Figure 8. PP2A inhibitor okadaic acid prevents CGN-induced increase in IL-8

Co-incubation with OA 5 nM with λ CGN 1 μ g/ml $\times$ 24 hours reduced the CGN-induced increase in IL-8 ($p < 0.001$, 1-way ANOVA with Tukey's post-test, $n=3$) by 62.5%. This finding suggests that the CGN-induced effect is mediated at least in part by PP2A. Baseline IL-8 secretion declined by ~32%.

A.

B.

Figure 9. In contrast to Tempol and CGN, Bcl10 silencing has no impact on Phospho-Hsp27
 In the cultured NCM460 cells, Bcl10 silencing by siRNA, CGN, and Tempol do not affect the total Hsp27 (**8A**). However, in contrast to the effects of Tempol and CGN, Bcl10 siRNA does not modify the phospho-Hsp27 content (**8B**), consistent with a distinct, non-ROS mediated mechanism for the observed effects associated with Bcl10 silencing.

Figure 10. Combined Bcl10 knockdown and ROS quenching by Tempol inhibit the CGN-induced activation of NFκB, phospho-IκBα, and IL-8

The CGN-induced increases in nuclear NFκB concentration (10A), phospho-IκBα (10B), and secreted IL-8 levels (10C) were reduced to less than baseline by the combination of Tempol and Bcl10 silencing, but neither Tempol nor Bcl10 silencing alone was sufficient to inhibit the CGN-induced increase. Differences from control values following CGN and CGN with Bcl10 siRNA or CGN with Tempol or CGN with Bcl10 siRNA and Tempol are statistically significant ($p < 0.001$, 1-way ANOVA with Tukey's post-test, $n = 3$).

Figure 11. Experimental model of CGN induced IL-8 secretion by in colonic epithelial cells
 The schematic drawing indicates two pathways of CGN-induced inflammation, through ROS and through Bcl10, that converge at the level of the IKK signalosome.

TABLE 1

Expression of Oxidation-Reduction Enzymes from cDNA Microarray

Enzyme	Fold-change	corrected p-value
Glutathione synthetase (glutamate cysteine ligase (GCLC)	1.7, 1.5	0.0009, 0.007
NADPH oxidase (NOX1)	-1.7, -1.6, -1.6, -1.6	0.009, 0.02, 0.09, 1
Selenoprotein S (SELS)	1.5	0.0001
Sulfiredoxin	1.3	1 ^a
Thioredoxin reductase 1	1.3	1 ^b

^a Increase in mRNA expression confirmed by QRT-PCR (p=0.002, unpaired t-test, two-tail; n=4).

^b uncorrected p-value = 0.00028