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Carrageenan induces interleukin-8 production through distinct Bcl10 pathway in normal human colonic epithelial cells

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Gastrointest Liver Physiol* 292: G829–G838, 2007. First published
November 9, 2006; doi:10.1152/ajpgi.00380.2006.—Carrageenan is a
high molecular weight sulfated polygalactan used to improve the
texture of commercial food products. Its use increased markedly
during the last half century, although carrageenan is known to induce
inflammation in rheumatological models and in intestinal models of
colitis. We performed studies to determine its direct effects on human
intestinal cells, including normal human intestinal epithelial cells from
colonic surgeries, the normal intestinal epithelial cell line NCM460,
and normal rat ileal epithelial cells. Cells were treated with high
molecular weight λ-carrageenan at a concentration of 1 μg/ml for
1–96 h. IL-8, IL-8 promoter activity, total and nuclear NF-κB, IκBα,
phospho-IκBα, and Bcl10 were assessed by immunohistochemistry,
Western blot, ELISA, and cDNA microarray. Increased Bcl10, nu-
clear and cytoplasmic NF-κB, IL-8 promoter activation, and IL-8
secretion were detected following carrageenan exposure. Knockdown
of Bcl10 by siRNA markedly reduced the increase in IL-8 that
followed carrageenan exposure in the NCM460 cells. These results
show, for the first time, that exposure of human intestinal epithelial
cells to carrageenan triggers a distinct inflammatory pathway via
activation of Bcl10 with NF-κB activation and upregulation of IL-8
secretion. Since Bcl10 contains a caspase-recruitment domain, similar
to that found in NOD2/CARD15 and associated with genetic predis-
position to Crohn's disease, the study findings may represent a link
between genetic and environmental etiologies of inflammatory bowel
disease. Because of the high use of carrageenan as a food additive in
the diet, the findings may have clinical significance.

inflammation; colon; NF-κB; IκBα

CARRAGEENANS ARE HIGH MOLECULAR weight sulfated polygalac-
tans derived from several species of red seaweeds (*Rhodo-
phyceae*). The most common forms of carrageenan (CGN) are
lambda (λ), kappa (κ), and iota (ι). λCGN has alternating
disaccharide units composed of D-galactose-2-sulfate and D-ga-
lactose-2,6-disulfate. κCGN is composed predominantly of
alternating D-galactose-4-sulfate and 3,6-anhydro-D-galactose
residues. ιCGN varies from κCGN by sulfation of the 3,6-
anhydro-D-galactose at the second carbon. Galactose residues
are joined by α-1,3 and β-1,4 linkages. CGNs resemble to
some extent the naturally occurring glycosaminoglycans owing
to their backbone composition of sulfated disaccharides. CGNs
are extracted from specific seaweeds, including *Gigartina*,
Chondrus, and *Eucheuma*, and are used by the food industry to
improve the texture of food products by acting to thicken,
stabilize, or emulsify dairy products, salad dressings, infant
formulas, processed meat, soy milk, and other food products
(37, 38). In addition to incorporation in foods, CGN has been
used as an ingredient in pharmaceuticals and personal care
products, such as toothpaste and cosmetics. The predominant
use of CGN has been in food preparations, where it is used
across a wide variety of food groups because of its ability to
substitute for fat and combine easily with milk proteins to
improve solubility and texture.

Earlier work in animal models, including rats, mice, rabbits,
guinea pigs, and monkeys, has demonstrated that CGN may
cause gastrointestinal pathology, including ulcerations and tu-
mors of the gastrointestinal tract (24, 37). In animals, dozens of
studies have used ingestion of CGN to induce gastrointestinal
ulcerations and inflammation that resemble inflammatory
bowel disease (IBD) (26, 27, 32). In addition, colorectal tumors
have been induced by exposure to CGN in animal models (18,
33, 39). These harmful effects of CGN are caused by both
native and degraded forms (37, 38). Studies on tumor promo-
tion, aberrant crypt cell formation, and cell proliferation in the
rat colon have been extensively reviewed (37, 16, 20). Rats
treated with the known tumor initiators azoxymethane or *N*-
methyl-*N*-nitrosourea and coadministration of CGN at 15 or
6% in the diet had increased incidence of colon tumors,
compared with animals treated with initiator alone (40). Un-
degraded CGN, administered in the diet of rats at a dose of 0.25
and 2.5% with azoxymethane injection, promoted the occur-
rence of aberrant crypt foci, consistent with premalignant
transformation (5). Cell proliferation, measured by increases in
thymidine kinase activity, increased severalfold in the colon of
rats when fed 5% degraded or undegraded CGN in solid food
or water (4, 9, 41). Responding to concerns about the safety of
CGN in the human diet, the European Union recently modified
their recommendations with regard to incorporation of CGN in
processed foods (8), endorsing a molecular weight limit of not
more than 5% CGN below 50 kDa into the specification, to
reduce exposure to degraded CGN.

CGN causes a reproducible inflammatory reaction and re-
mains a standard chemical for examining acute inflammation
and effects of anti-inflammatory drugs (22, 25). CGN-induced
edema of rat hind paw has been an extensively used model of
inflammation (42). In addition, CGN has been used in intestinal
models of inflammation in animals and to introduce inflamma-
tion in extracolonic sites, including pleural space and perito-
neum (7, 26, 27, 28, 32, 37, 38). These models have often used

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higher doses of CGN than are normally consumed in the Western diet to induce inflammation.

Although the effects of CGN on intestine have been extensively studied in animal models, parallel studies on human intestinal epithelium have not been performed. In this report, we present the effects of exposure of NCM460 cells, a nontransformed human intestinal epithelial cell line, primary colonic epithelial cells, and fresh colonic tissues, to a small dose of λCGN (1 μg/ml). This dose is lower than the anticipated daily exposure from CGN in the Western diet. Measurements of ingestion in the 1970s reported average intake of CGN to be ~100 mg/day, and subsequent estimates have ranged to as high as 3.6 g/day (38). In intestinal content of 1.5 μl, ingestion of 100 mg/day represents an exposure of the human intestine to about 67 μg/ml, far higher than we have used in these experiments. We demonstrate that λCGN at low dose (1 μg/ml) triggers an inflammatory cascade in normal human colonic epithelial cells and ex vivo intestinal cells within 24 h post-CGN exposure, resulting in significantly increased IL-8 production. The increase in IL-8 is dependent on activation of NF-κB and increased Bcl10. Although Bcl10 is linked with development of lymphomas and inflammation through NF-κB activation in lymphocytes, this is the first report of its association with inflammation in human intestinal cells. Since Bcl10 contains a caspase-recruitment domain (CARD) and mutations in NOD2/CARD15 have been associated with predisposition to Crohn's disease, the CGN-induced activation of a Bcl10-mediated pathway of intestinal inflammation may provide a link between genetic and environmental causes of IBD (15, 31).

MATERIALS AND METHODS

Cell culture and CGN exposure. Cells of the nontransformed human colonic epithelial cell line NCM460 were grown in M3:10 medium (INCELL, San Antonio, TX) and were maintained at 37°C in a humidified, 5% CO_2 environment (27a). For experiments, confluent cells in cell culture flasks were trypsinized and seeded into either 6-well or 12-well plates at a cell density of 2×10^4 cells/ml. At 50–60% confluency, cells were treated with CGN at a concentration of 1 μg/ml for different time periods.

Normal rat ileal epithelial cells (CRL-1589) were obtained from American Type Culture Collection (Manassas, VA) and grown under recommended conditions with DMEM and 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 0.1 unit/ml bovine insulin, and fetal bovine serum 5% at 37°C and 5% CO_2. Confluent cells in cell culture flasks were trypsinized and seeded into either 6-well or 12-well plates and treated in a similar fashion as the NCM460 cells.

Cell and tissue preparations were exposed to type IV λCGN, an essentially pure λ form with weight average molecular weight of 1×10^6. CGN was dissolved in ddH₂O at a concentration of 10 mg/ml and aliquoted. The κ- and ιCGNs (Sigma) were similarly prepared. In experiments, a concentration of 1 μg/ml ($10^{-4}\%$) was used, unless otherwise stated, for time periods ranging from 1 to 96 h. Osmolarity and pH of the media were measured and found to be unaffected by addition of λCGN. Control samples were maintained under similar conditions in all instances, except cells were not exposed to λCGN. TNF-α was obtained from Sigma.

Ex vivo colonic tissue preparations. Deidentified 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. Access to surgical specimens was facilitated by the Tissue Bank of the University of Illinois at Chicago Hospitals and Clinics and the Department of Pathology. Patients consented to donate

tissue to the Tissue Bank for research purposes. Normal tissue that was several centimeters away from any macroscopic lesion was obtained under sterile conditions, and small tissue samples were scraped from the underlying muscle. The samples were placed in DMEM medium with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Small tissue fragments of 2 mm × 2 mm were obtained by mincing the fresh samples. These were placed in 12-well tissue culture plates. Untreated control samples and comparable samples exposed to λCGN (1–10 μg/ml) for 1–24 h were incubated in a 37°C, 5% CO_2, humidified incubator. Spent media were collected from triplicate control and treated samples. Tissue samples were lysed for protein determinations, and other samples were deep frozen for subsequent immunohistochemistry.

Some of the mucosal tissue that had been scraped from the underlying muscle and minced was placed in a flask with 50 ml DMEM with 10% FBS and penicillin (100 U/ml) and streptomycin (100 μg/ml) and 50 ml of an enzyme digestion mixture [1 mM calcium, 3 mM glucose, 4% bovine serum albumin, 2 mg/ml collagenase (Worthington, Type IV) in DMEM], and incubated at 37°C in 5% CO_2 for 6 h with periodic stirring. Pellets were obtained following serial centrifugations at 800 rpm for 5 min and plated in T25 flasks with M3:10 (INCELL) medium or DMEM with 10% FBS and Pen-Strep antibiotics. Medium was changed after 24 h; spent medium was centrifuged at 800 rpm for 5 min, and the cell pellet was resuspended in medium and plated in T25 flasks. Medium was changed at 3- to 4-day intervals following attachment. Cells were photographed by use of the Moticam 2000 camera and the Motic AE31 inverted microscope. On examination, cells appear nearly all epithelial.

Preparation of nuclear extracts and whole cell lysate. Nuclear extracts were prepared as described previously (12). Briefly, cells were washed twice with PBS and transferred to an Eppendorf tube. Cells were lysed in lysis buffer [in mM: 10 HEPES (pH 7.9), 10 KCl, 1.5 MgCl₂, 0.1 EDTA, 1 DTT, and 1 PMSF], kept on ice for 10 min, and then dounced 10 times on ice in a Dounce homogenizer. The nuclear pellet obtained by centrifugation was washed with lysis buffer and resuspended in 50–80 μl of nuclear extraction buffer [in mM: 20 HEPES (pH 7.9), 400 NaCl, 1.5 MgCl₂ 1 EDTA, 1 DTT, 1 PMSF, with 25% glycerol] and rotated at 4°C for 3 h. Supernatant (nuclear extract) obtained by centrifugation was stored at −80°C until further use. To prepare the whole cell lysate, cell monolayers were washed with PBS and transferred to Eppendorf tubes. Cells were suspended in a suitable volume of complete lysis buffer [in mM: 10 Tris·HCl pH 7.5, 150 NaCl, 5 EDTA, 1 PMSF, 1% Triton X-100, 1× protease inhibitor cocktail (Roche)], incubated on ice for 30 min, and sonicated twice for 20 s. Cell preparations were spun at 13,500 g for 20 min at 4°C, and the supernatant (cell lysate) was collected and stored at −80°C .

Western blot. Proteins in the nuclear extracts or in the whole cell lysates were separated by SDS-PAGE on a 12% gel. Proteins were transferred to a nitrocellulose membrane (Amersham Biosciences) and probed with the indicated antibodies [human anti-NF-κB, IκBα, and Bcl10 (Santa Cruz Biotechnology, Santa Cruz, CA)] and anti-phospho-IκBα (Cell Signaling). Immunoreactive bands were visualized using the ECL detection kit (Amersham).

cDNA microarray analysis. cDNA microarray was performed using the Affymetrix U133 plus 2.0 system, following extraction of total RNA by RNeasy Mini Kit (Qiagen). RNA quality was checked by analysis of 18S and 28S fractions on agarose gel. The microarrays were hybridized and scanned according to protocol. Results were analyzed with Affymetrix software to generate gene expression values and expression differentials by S+Array Analyzer. Signal intensities of biological and technical replicates were summarized and normalized by using microarray suite software (MAS) for background correction, for mismatch correction, and quantile normalization. Pairwise comparisons of control vs. treated samples were averaged. Fold

changes and *P* values, including Bonferroni corrected *P* values, were calculated for the observed differences in signals.

Immunohistochemistry of ex vivo colonic tissue. Immunohistochemistry for NF-κB and Bcl10 in fresh human colonic tissue samples exposed to CGN and controls was performed on colonic specimens after 1 and 2 h of exposure to λCGN. Representative tissue samples were frozen after 1 and 2 h of exposure or no exposure and deep frozen. Subsequently, control and treated tissue samples were pro-

cessed simultaneously. Five-micrometer sections were cut by microtome. Tissue sections on the slides were hydrated, and antigen retrieval was performed with DakoTarget Retrieval 10× citrate buffer solution (DakoCytomation) for 20 min in a steamer at 95°C . Slides were then equilibrated to room temperature in the same solution for 20 min, rinsed in dH$_2$O, placed in buffer solution for 15 min, placed in 3% H$_2$O$_2$ for 10 min, and rinsed in buffer, and protein was blocked for 10 min. Slides were incubated with the appropriate primary antibody
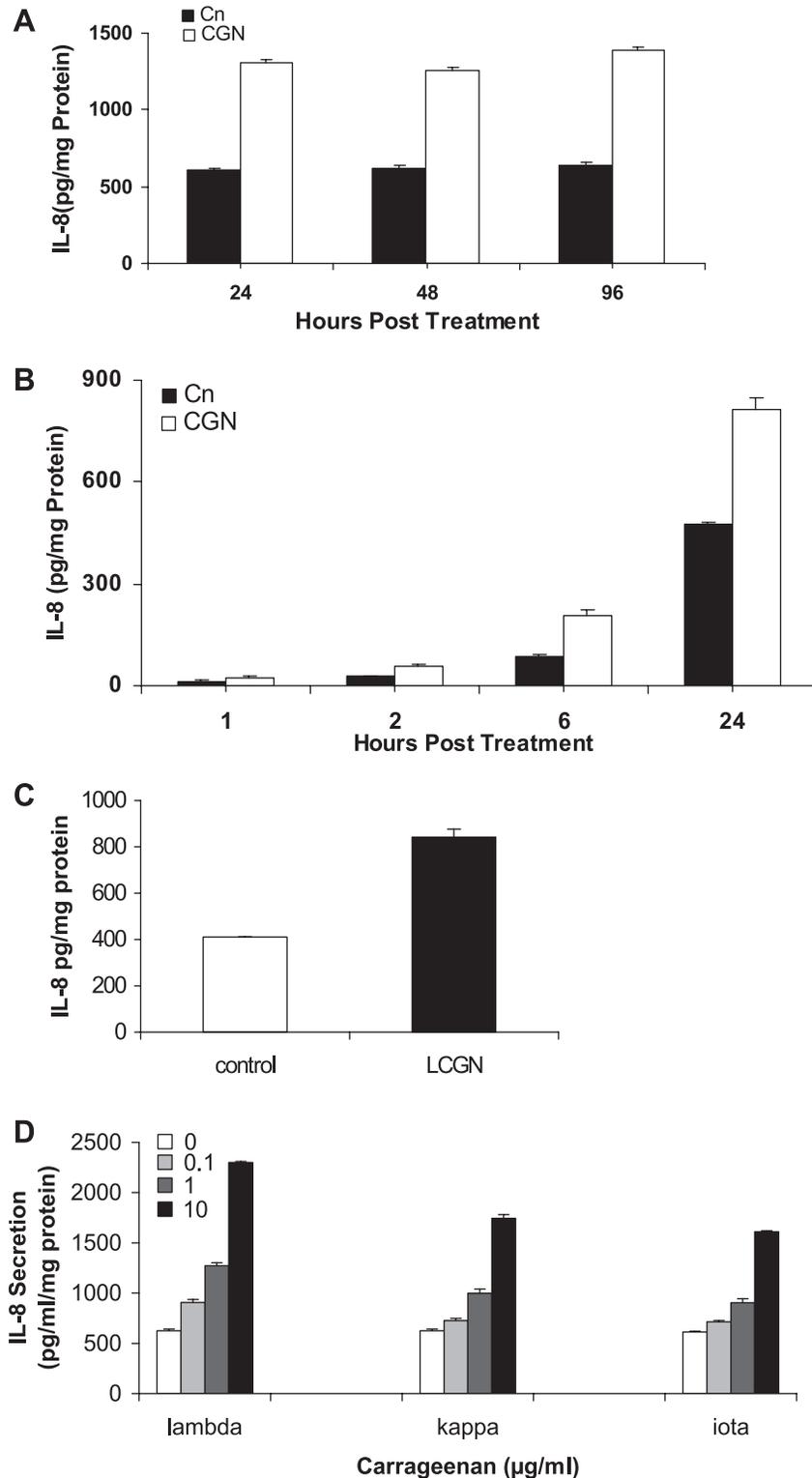

Fig. 1. Carrageenan (CGN) induces increased IL-8 secretion in NCM460 cells, ex vivo human colonic tissue, and primary human colonic epithelial cells. *A*: NCM460 cells at 50–60% confluency were treated with λCGN (1 μg/ml) for 24–96 h. *B*: ex vivo human colonic epithelial tissues were treated with λCGN 10 μg/ml for 1–24 h. IL-8 concentrations in the spent media were determined by ELISA as described in MATERIALS AND METHODS and normalized with protein of cell lysates. Results are means (SD) of 3 independent experiments performed with technical duplicates. Statistical significance was determined by 1-way ANOVA followed by Tukey-Kramer multiple comparison test. Differences between the control (Cn) and CGN groups are statistically significant with *P* < 0.001 for all time points. *C*: IL-8 secretion in the primary colonic epithelial cell cultures was 411.2 (0.6) and increased by 105% to 842.5 (34.4) following exposure to λCGN (LCGN) for 24 h. *D*: IL-8 secretion (pg·ml^{-1}·mg^{-1} protein (SD) following exposure to different concentrations of λ-, κ-, and ι-CGN in NCM460 cells from baseline, unstimulated value of 624.6 (17.8) pg·ml^{-1}·mg^{-1} protein. Results are obtained from biological triplicates with technical duplicates of each. Standard deviations of IL-8 determinations are ≤4.1%.

[NF-κB p65 (DakoCytomation) 1:100 dilution or Bcl10 (Santa Cruz) 1:50 dilution], and NF-κB antibody was incubated for 1 h and Bcl10 was incubated overnight. Slides were rinsed in buffer, and an anti-mouse secondary antibody (DakoCytomation EnVision mouse monoclonal kit) was applied for 30 min. Slides were rinsed in buffer, then treated with 3,3′-diaminobenzidine for color detection, rinsed in H₂O, dehydrated, coverslipped with Permount, and photographed with Nikon imaging system. No enhancement or modification of the images was performed.

ELISA for IL-8 in NCM460 cells and normal colonic excised tissue. The secretion of IL-8 in the spent media of control and CGN-treated NCM460 cells, primary colonic epithelial cells, and ex vivo intestinal tissue was measured by using the Quantikine ELISA kit for human IL-8 (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Range of detection of the standard curve is 31.2–2,000 pg/ml with a sensitivity of 1.5–7.5 pg/ml. Briefly, the IL-8 in the medium was captured into the wells of a microtiter plate precoated with specific anti-IL-8 monoclonal antibody. Immobilized IL-8 was

then detected by a horseradish peroxidase (HRP)-conjugated secondary antibody with the use of the chromogenic substrate hydrogen peroxide/tetramethylbenzidine (TMB). Color intensity was read at 450 nm with a reference filter of 570 nm in an ELISA plate reader (SLT, Spectra). The IL-8 concentrations were extrapolated from a standard curve plotted by using known concentrations of IL-8. The sample values were normalized with total cell lysate protein content (BCA protein assay, Pierce) and expressed as picograms per milligram protein.

ELISA for phospho-IκBα in NCM460 cells and primary human intestinal epithelial cell cultures. Phospho-IκBα was determined in the NCM460 cells following Bcl10 knockdown by small interfering RNA (siRNA) using the PathScan sandwich ELISA kit (no. 7355, Cell Signaling) that detects phosphorylation of serine 32. The method utilizes a solid phase sandwich ELISA in which a mouse monoclonal antibody against IκBα has been coated onto the microwells of a 96-well plate. After incubation with cell lysates and capture by the coated antibody, the wells are extensively washed and a second
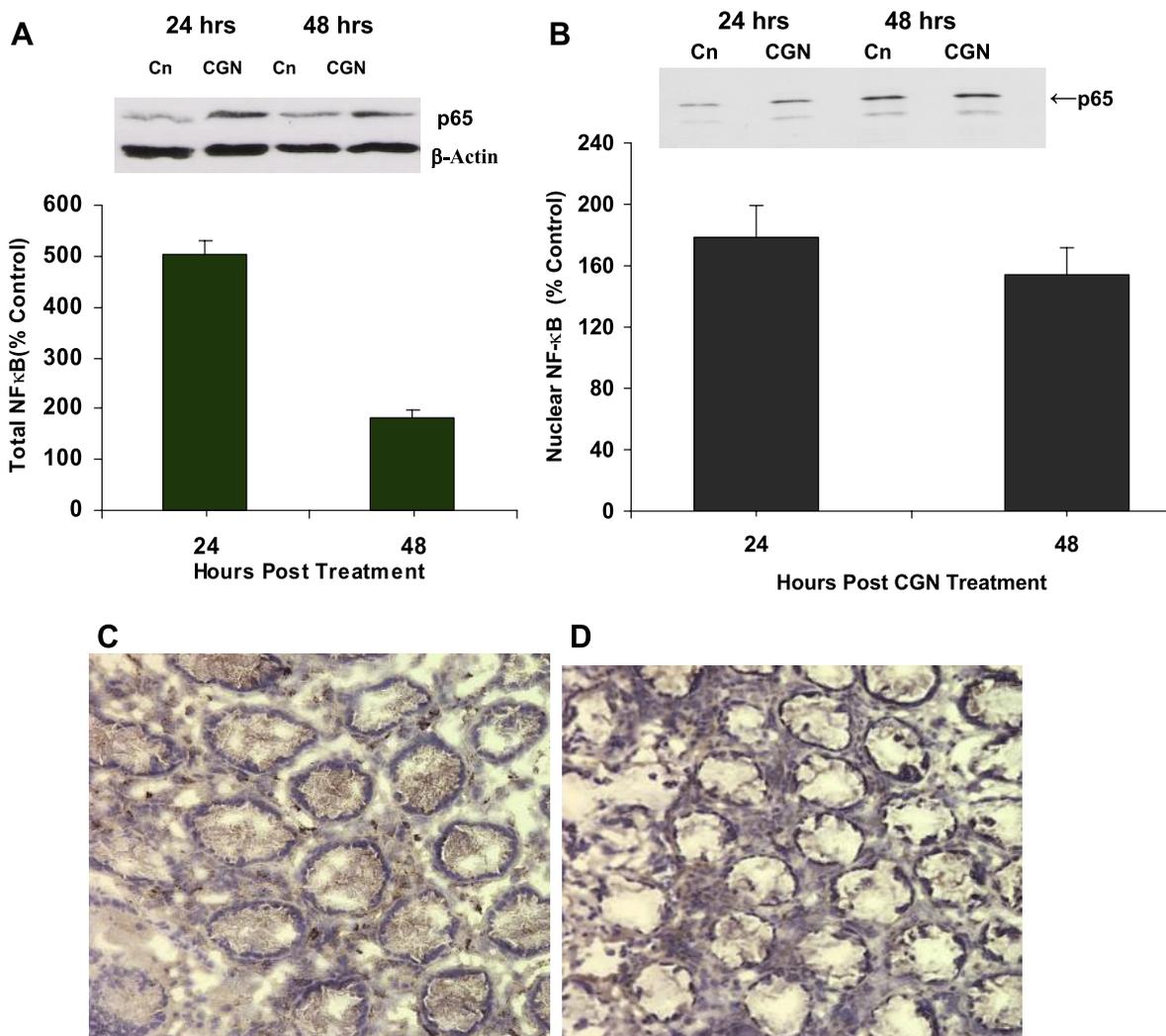

Fig. 2. Carrageenan treatment increases total and nuclear NF-κB in NCM460 cells and immunohistochemical staining for NF-κB in ex vivo human colonic tissue. NCM460 cells grown in 6-well plates were treated with λCGN (1 μg/ml) for the indicated periods. Whole cell lysates or nuclear extracts of NCM460 cells were prepared as described in MATERIALS AND METHODS. Total cell proteins of NCM460 lysates (*A*) or nuclear proteins of NCM460 (*B*) were subjected to SDS-PAGE and probed with human anti-p65 NF-κB antibody. Immunoblot for β-actin (*A*) served as loading control. Cn, control; CGN, λCGN treated. Representative blots of 3 independent experiments are shown. The percent increase in p65 over control at 24 and 48 h after CGN treatments was measured by densitometric scanning of 3 independent immunoblots. Densitometric differences are statistically significant for both nuclear and cytoplasmic NF-κB ($P < 0.001$, paired *t*-test). *C* (CGN treated) and *D* (control): immunohistochemical staining for NF-κB in excised normal human colonic tissue treated with λCGN at a concentration of 10 μg/ml for 2 h.

antibody is added to detect the captured phospho(Ser 32)-IκBα protein. An HRP-linked anti-rabbit antibody recognizes the bound detection antibody and hydrogen peroxide/TMB, is added for color development. The magnitude of the optical density is proportional to the quantity of the phospho-IκBα protein.

Measurement of IL-8 promoter activity by luciferase assay. IL-8 promoter in pGL2 vector was a gift from Gary Wu (University of Pennsylvania). NCM460 cells at 80–90% confluency were transiently transfected with the IL-8 promoter plasmid or empty vector in 24-well plates using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol. At 24 h after transfection, fresh medium or medium containing 1 μg/ml CGN was added. For studies with NF-κB inhibitor, cells were pretreated for 1 h with the NF-κB inhibitor caffeic acid phenylether ester (CAPE) and then treated with λCGN (1 μg/ml) for 24 h before being assayed for luciferase activity

CGN-treated and control cell preparations were washed with PBS and lysed by alternate freezing and thawing in 50 μl of reporter lysis buffer (Promega). Twenty microliters of the lysate were used for firefly luciferase assays with luciferase assay reagent (Promega) in a luminometer. Relative luciferase unit (RLU) values obtained from the assay were normalized by the protein and expressed as RLU per milligram protein (3).

Bcl10 knockdown by siRNA. siRNA for Bcl10 was obtained commercially (Qiagen) and found to effectively reduce Bcl10 production in NCM460 cells by Bcl10 Western blot. No band for Bcl10 was visible following siRNA treatment in the NCM460 cells. Bcl10 siRNA (150 ng; 0.6 μl) in 100 μl serum-free culture medium was mixed with 12 μl of HiPerfect Transfection Reagent (Qiagen) by vortexing, maintained at room temperature for 10 min, then added dropwise onto the NCM460 cells in a six-well culture plate with a cell density of about 2.5×10^5 per well. Cells were swirled for uniform distribution of the transfection complex and the transfected cells were incubated at 37°C and 5% CO_2. Subsequently, 24 h later cells were exposed to λCGN at a concentration of 1 μg/ml for an additional 24 h. Media were collected and IL-8 secretion in the spent media was measured by ELISA.

Statistical analysis. Statistical analysis was performed using GraphPad InStat Software (GraphPad Software, San Diego, CA). One-way ANOVA tests with Tukey-Kramer multiple comparison test for posttest correction were used for analysis of IL-8 ELISA test results. Paired *t*-tests were used to compare the results of densitometry of Western blots between treated and control samples. A *P* value of <0.05 is considered statistically significant. ELISA results are means (SD) of three independent experiments with technical replicates of each measurement.

RESULTS

IL-8 secretion by NCM460 cells, ex vivo colonic tissue, and primary colonic epithelial cells after CGN exposure. Secretion of IL-8 increased ~2-fold in NCM460 cells, ex vivo colonic tissue and primary human intestinal epithelial cells (IEC), following λCGN exposure (Fig. 1, *A–C*). Increases occurred by 24 h in the NCM460 cells and remained elevated at 96 h. Increases were seen at 1, 2, 6, and 24 h in the fresh tissue samples and at 24 h in the primary human IEC (Fig. 1*C*). Results presented in Fig. 1*B* indicate that at 1 h CGN treatment led to an 80% increase (from 14.2 to 25.7 pg/mg protein) in IL-8 in the ex vivo colonic tissue. By 2 h, IL-8 secretion increased 100%, and at 4 h, IL-8 had increased by 250%. By 24 h, the control sample value has also increased substantially, to 476.0 pg/mg protein, reflecting the generalized cell stress that has occurred. λCGN-treated colonic tissue continued to demonstrate significantly higher IL-8 (816.7 pg/mg protein) than the control. In the primary human IEC, λCGN (1 μg/ml)

induced 105% increase in IL-8 secretion over control at 24 h. Results were statistically significant at the $P < 0.001$ level (1-way ANOVA, Tukey-Kramer posttest) at all time points.

Figure 1*D* indicates that IL-8 secretion rose linearly in response to increasing concentration of undegraded λ-, κ-, or ιCGN. The IL-8 response to exposure to 1 μg/ml for 24 h was greatest for λ ($1,273.0 \pm 29.9$ pg·ml^{-1}·mg^{-1} protein), next for κ (997.2 ± 41.7 pg·ml^{-1}·mg^{-1} protein), followed by ι (903.1 ± 41.4 pg·ml^{-1}·mg^{-1} protein), from a baseline, unstimulated IL-8 secretion of 624.6 ± 17.8 pg·ml^{-1}·mg^{-1} protein. In comparison, the IL-8 response to TNF-α at the supraphysiological concentration of 10 pg/ml was 942 ± 31 pg·ml^{-1}·mg^{-1} protein.

Exposure to CGN increased NF-κB levels in NCM460 cells. NCM460 cells at 50–60% confluency were treated with λCGN (1 μg/ml) for 24 and 48 h, after which cells were harvested to make whole cell lysates or nuclear extracts. As shown in Fig. 2, there was significant increase in total (*A*) and nuclear (*B*) levels of the p65 subunit of NF-κB at both time points after λCGN treatment. Increase in total NF-κB in response to CGN was approximately fivefold at 24 h; increase in nuclear NF-κB was approximately twofold at both time points. Densitometry demonstrated statistically significant differences between treated and control samples at all time points with $P < 0.001$ (paired *t*-test). We also found a significant increase in nuclear p65 in rat IEC, upon exposure to λ-CGN for 24 and 48 h (results not shown).

Immunohistochemical staining for NF-κB in the ex vivo colonic samples demonstrated marked increase in intensity of brown staining for NF-κB in the CGN-treated samples (Fig. 2*C*) vs. control (Fig. 2*D*). Samples were exposed to 10 μg/ml λCGN for 2 h. Treated cells appear to be somewhat swollen, relative to the untreated cells.

Exposure to CGN causes increased phosphorylation of IκBα. In the NF-κB activation pathway, IKK complex phosphorylates IκB, leading to release of NF-κB that was bound to IκB. IκB is targeted for proteasomal degradation, and nuclear translocation of active NF-κB occurs (2). Therefore, it was of interest to monitor the changes in total and phospho-IκBα, one of the major IκB isoforms, in response to CGN treatment. As shown in Fig. 3*A*, CGN treatment of NCM460 cells for 24 and 48 h caused increased phosphorylation of IκBα, compared with untreated cells. Total IκBα levels at both time points were less in CGN-treated cells compared with control (Fig. 3*B*),
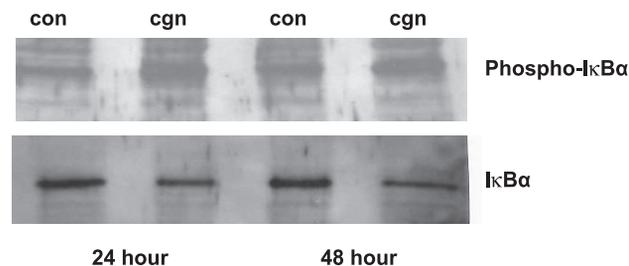

Fig. 3. Carrageenan induces increased IκBα phosphorylation in NCM460 cells. *A*: NCM460 cells grown in 6-well plates were treated with λCGN (1 μg/ml) for 24–48 h. Total proteins in the whole cell lysates were subjected to SDS-PAGE and probed with human anti-phospho-IκBα antibody. *B*: the same blot was stripped and probed with human anti-IκBα antibody, to compare the relative levels of phosphorylated and total IκBα in control (con) and CGN-treated (cgn) cells. Increase in phosphorylated IκBα and decline in total IκBα at both 24 and 48 h are found.

since phosphorylation leads to proteasomal degradation of IκB. Increase in phospho-IκBα after exposure to λCGN was also determined by ELISA (see Fig. 7).

Increased Bcl10 mRNA and protein expression in response to λCGN treatment. Bcl10, a CARD-containing protein, has been shown to be an activator of the NF-κB pathway in lymphocytes (6, 11). cDNA microarray analysis showed significant upregulation of Bcl10 in NCM460 cells after exposure to CGN for 96 h, with highly significant *P* values following Bonferroni correction and fold changes of 2.0 in three different gene identifiers (IDs) associated with Bcl10 (results not shown). Figure 4*A* demonstrates increases in Bcl10 levels by Western blot of the NCM460 whole cell lysate at 24 and 48 h after exposure to λCGN (1 μg/ml) vs. control, consistent with a role of Bcl10 in the activation of the NF-κB pathway after CGN treatment. Bcl10 was normalized by comparison to

β-actin. Densitometry results are presented in Fig. 4*B* and demonstrate statistically significant increase ($P < 0.001$ by paired *t*-test) in Bcl10 following λCGN exposure. Figure 4*C* demonstrates a representative Western blot of Bcl10 following exposure to κ-, λ-, and ιCGN (1 μg/ml) at 24 h. Again, increases in Bcl10 vs. control are evident.

Immunohistochemical staining for Bcl10 in ex vivo colonic tissue samples treated for 2 h with 10 μg/ml λCGN is presented in Fig. 4, *C* (λCGN-treated) and *D* (control). Increased brown intensity is characteristic of increase in Bcl10 protein.

CGN-induced increase in IL-8 secretion in NCM460 cells requires NF-κB pathway and is transcriptionally mediated. To examine the mechanism(s) of induction of IL-8 secretion by CGN, we examined the effect of inhibition of the NF-κB pathway. Exposure of the NCM460 cells to 50 μM CAPE, a specific inhibitor of NF-κB activation (29), reduced IL-8 se-
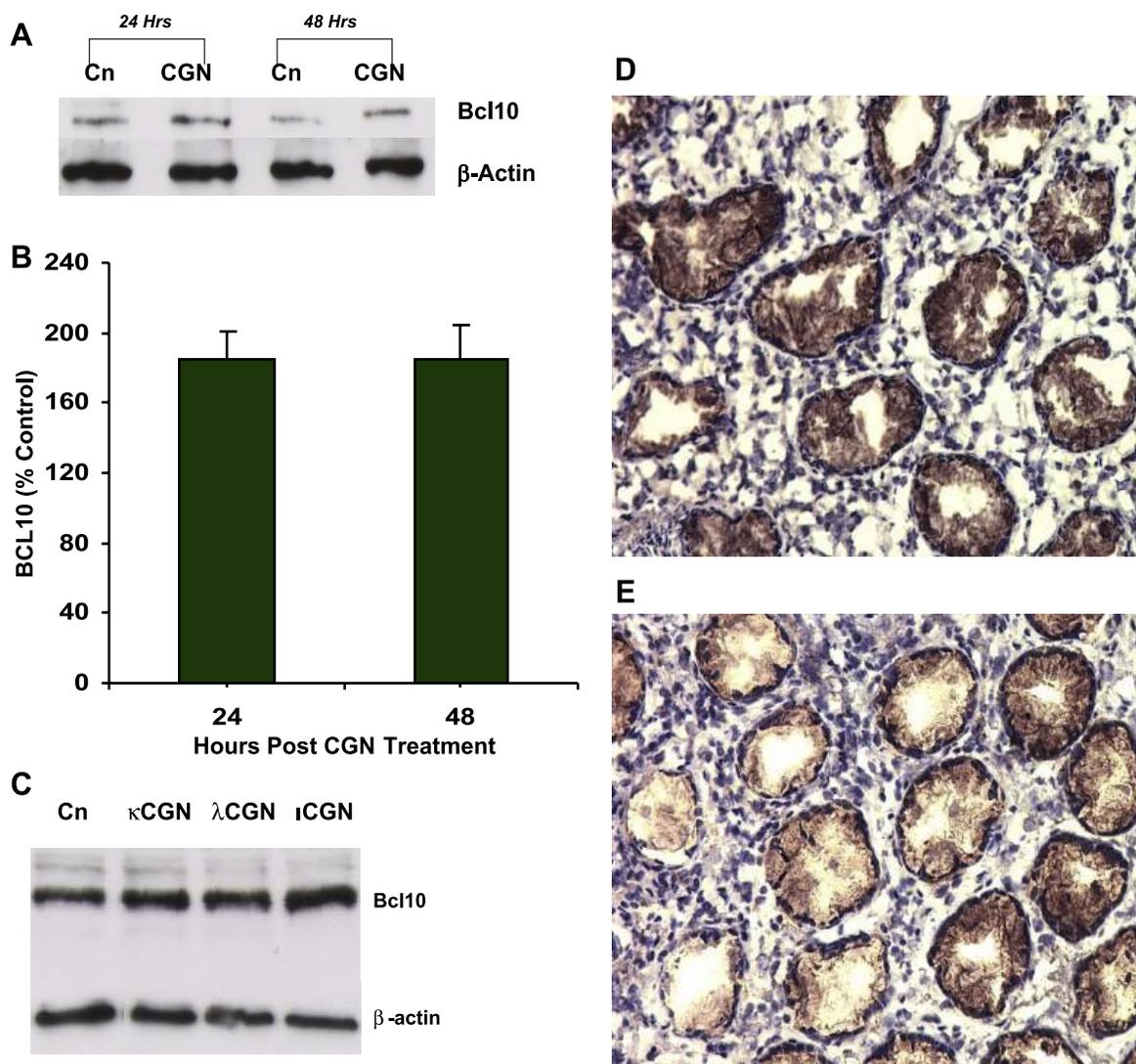

Fig. 4. Carrageenan exposure increases Bcl10 in NCM460 cells and in ex vivo colonic tissue. NCM460 cells grown in 6-well plates were treated with λCGN (1 μg/ml) for 24–48 h. Total proteins in the whole cell lysates were subjected to SDS-PAGE and probed with human anti-Bcl10 antibody. The same blot was stripped and probed with human anti-β-actin antibody to demonstrate equal loading. *A*: representative blot of 3 independent experiments that demonstrates increase in Bcl10 following exposure to λCGN at a concentration of 1 μg/ml for 24 and 48 h, compared with control. *B*: % increase of the mean (SD) determinations of Bcl10 in λCGN-exposed vs. control NCM460 cells at 24 h by densitometry of 3 independent immunoblots. *C*: representative Western blot of Bcl10 following exposure to κ-, λ-, and ιCGN (1 μg/ml) for 24 h, normalized by comparison to β-actin. *D* (CGN-treated) and *E* (control): immunohistochemical staining for Bcl10 in excised normal human colonic tissue treated with λCGN at a concentration of 10 μg/ml for 2 h.

A

B

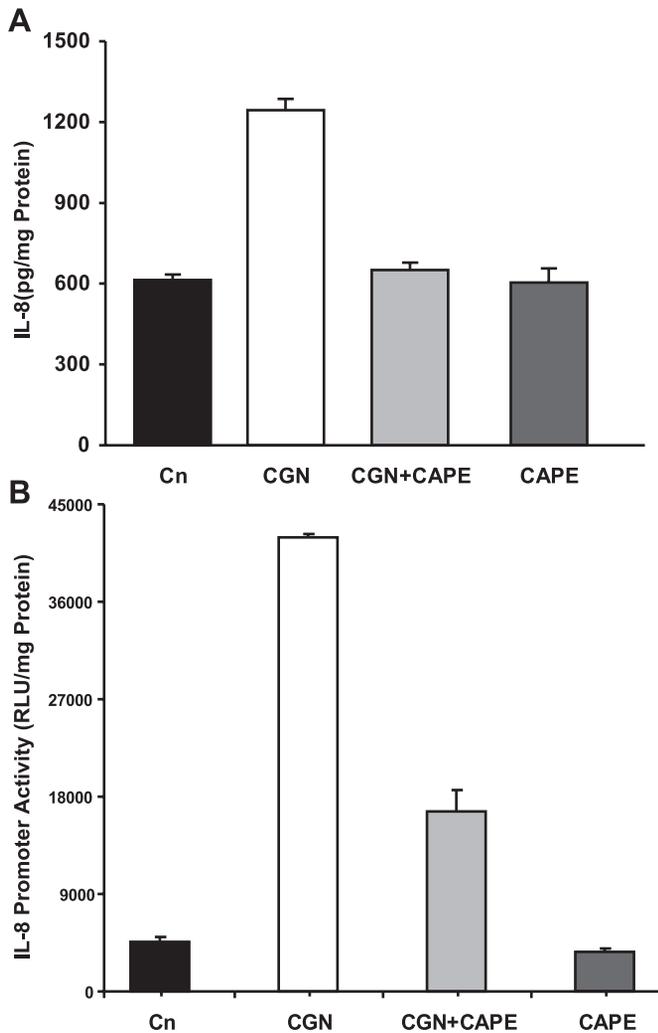
Fig. 5. λCGN-induced IL-8 secretion in NCM460 cells is NF-κB pathway dependent and transcriptionally regulated. *A*: NCM460 cells were treated with λ-carrageenan (1 μg/ml) for 24 h with or without 50 μM caffeic acid phenylether ester (CAPE), the NF-κB pathway inhibitor. IL-8 concentrations in the spent media were determined by ELISA as described in MATERIALS AND METHODS and normalized by the corresponding cell lysate total protein. Results are means (SD) of 3 independent experiments performed with technical replicates. Statistical significance was determined by 1-way ANOVA followed by a Tukey-Kramer multiple comparison test. Differences between the groups, Cn vs. CGN and CGN vs. CGN+CAPE, are statistically significant with $P < 0.001$. *B*: NCM460 cells were transfected with the IL-8 promoter construct in pGL2 vector using Lipofectamine 2000 reagent. After 24 h, cells were treated with the NF-κB inhibitor CAPE (50 μM), and λCGN (1 μg/ml) with or without CAPE for another 24 h. Luciferase activity was determined, values were normalized with total cell protein, and promoter activity was expressed as relative luciferase units (RLU)/mg protein. Data are means (SD). Statistical significance was determined by 1-way ANOVA followed by Tukey-Kramer posttest. Differences between the groups, Cn vs. CGN and CGN vs. CGN+CAPE are statistically significant with $P < 0.001$.

cretion to control levels (Fig. 5*A*), consistent with dependence of the CGN-induced increase in IL-8 secretion on NF-κB activation.

To determine whether the induction of IL-8 production by CGN involves transcriptional regulation, we examined the effect of CGN exposure on the IL-8 promoter activity, using a luciferase reporter assay. As shown in Fig. 5*B*, CGN treatment (1 μg/ml) for 24 h caused ~10-fold increase in IL-8 promoter

activity (RLU) compared with control. In the presence of 50 μM CAPE, there was marked reduction in the stimulation of IL-8 promoter activity by CGN. These results suggest that stimulation of IL-8 production by CGN requires activated NF-κB and is transcriptionally mediated.

Bcl10 knockdown markedly reduces CGN-induced increase in IL-8 secretion in NCM460 cells. To further evaluate the role of Bcl10 in the CGN-induced increase in IL-8 secretion, we exposed cells to siRNA for Bcl10 and measured the effect on IL-8 secretion in the NCM460 cells. Results are presented in Fig. 6 and demonstrate marked decline in the λCGN-induced IL-8 secretion following Bcl10 knockdown. This result suggests a pathway of CGN-induced IL-8 activation that requires Bcl10 activation. Results for changes in IL-8 following Bcl10 knockdown are statistically significant at the $P < 0.001$ level (1-way ANOVA and Tukey-Kramer posttest).

CGN-induced increase in phospho-IκBα decreases following Bcl10 knockdown. Measurements of phospho-IκBα were performed using an ELISA assay (Cell Signaling) based on relative optical densities (Fig. 7). Marked decline (50%) in the CGN-induced increase of phospho-IκBα occurs following knockdown of Bcl10 by siRNA. However, Bcl10 knockdown does not lead to decline in the baseline level of phospho-IκBα. These findings demonstrate that the Bcl10 effect on IL-8 activation is upstream of NF-κB. The differences between control vs. λCGN-treated and λCGN-treated vs. Bcl10si + λCGN-treated are statistically significant ($P < 0.001$, 1-way ANOVA with Tukey-Kramer posttest).

A

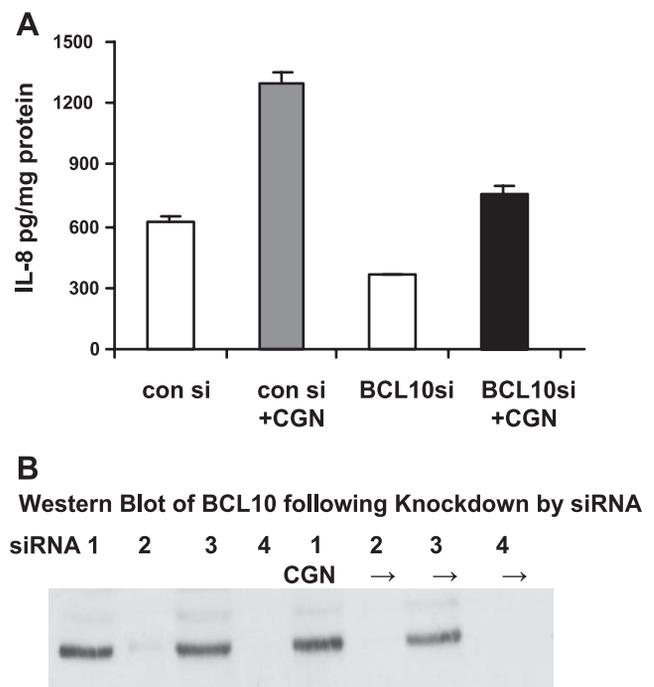# B

Western Blot of BCL10 following Knockdown by siRNA

Fig. 6. Knockdown of Bcl10 in NCM460 cells reduces CGN-induced increase in IL-8 secretion. *A*: knockdown of Bcl10 by small interfering RNA (siRNA) markedly reduces the λCGN-induced increase in IL-8 secretion. si, siRNA; CGN, λCGN 1 μg/ml × 24 h following Bcl10 knockdown. Changes in IL-8 are statistically significant at the $P < 0.001$ level (1-way ANOVA, Tukey-Kramer posttest) following knockdown of Bcl10. *B*: Western blot presents effectiveness of knockdown of Bcl10 by siRNA in *lanes 2* and *4*. Knockdown following CGN is shown in *lanes 5–8* and demonstrates effectiveness of Bcl10 knockdown by no. 2 and no. 4 siRNAs.
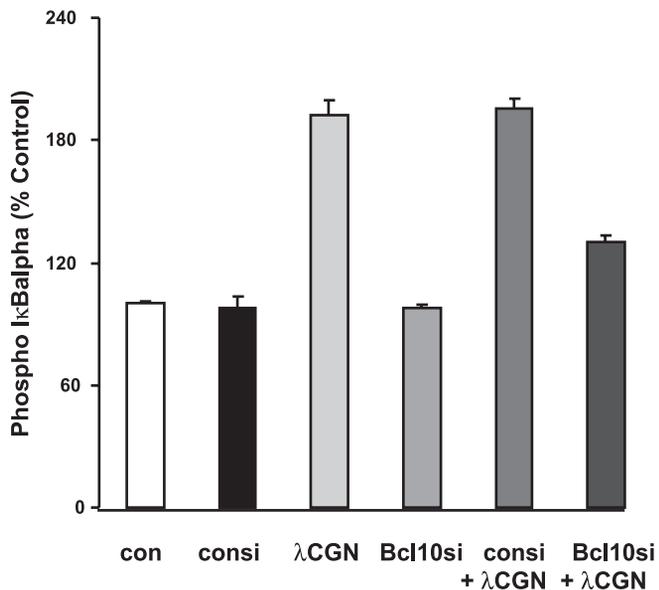
Fig. 7. Phospho-IκBα following Bcl10 knockdown in NCM460 cells by ELISA. The increase in phospho-IκBα that follows exposure to λCGN is reduced 50% following Bcl10 knockdown by siRNA. The differences between control vs. λCGN-treated and λCGN-treated Bcl10si + λCGN-treated are statistically significant ($P < 0.001$, 1-way ANOVA with Tukey-Kramer multiple comparison test).

DISCUSSION

The intestinal epithelium, in addition to its important roles in nutrient absorption and in entry of many invasive pathogens, is also an active participant in the inflammatory response due to immunological and other agents. In recent years, extensive evidence has accumulated documenting that IEC play a central role in orchestrating gut immune responses to both exogenous and endogenous antigens. In addition to the classical inflammatory signals presented by bacteria, bacterial products, or immune cell-derived cytokines, other stimuli have also been shown to elicit upregulation of cytokine response by the IEC (10, 13, 21, 30). CGN, a sulfated polygalactan derived from seaweeds and extensively used as a food additive, has been widely used to induce inflammation in experimental models of colitis in animal models (37, 38). In multiple studies, ingestion of CGN induced gastrointestinal ulcerations and inflammation that resemble IBD (26, 27, 32). Despite the fact that use of CGN as a food additive has increased markedly over the last half century in the Western diet, this study provides the first report of the inflammatory response of human IEC to CGN exposure. The experimental model of inflammation in human IEC that we propose is presented in Fig. 8.

NF-κB is a key determinant of the intestinal epithelial inflammatory cascade and occupies a central role in the transcriptional activation of proinflammatory genes (19). In the present study, the increased levels of NF-κB in NCM460 cells and in ex vivo colonic tissue, after exposure to low doses of λCGN for short duration, suggest that there may be an important role for CGN in induction of human intestinal inflammation. Previously, CGN-induced lung injury in mice has been associated with activation of NF-κB (25). NF-κB is composed of different dimers belonging to Rel family members including p50, p52, Rel A (p65), Rel B, and cRel (1). It is believed that the variability in the composition of NF-κB dimers may con-

tribute to the specificity of gene regulation, as a particular NF-κB sequence may bind certain NF-κB complexes but not all of them (23). We found increased levels of p65 after CGN exposure both in human colonic NCM460 cells and in rat IEC. In unstimulated cells, NF-κB dimer is sequestered in the cytosol as an inactive complex with IκB. In response to various stimuli, IκB is phosphorylated and degraded by the proteasome (34). The resulting free NF-κB is then translocated to the nucleus, where it can bind to promoter regions of target genes (34). Our results also demonstrated that CGN treatment caused increased phosphorylation of IκBα and a concomitant decrease in total IκBα, attributable to proteasomal degradation of IκBα following phosphorylation. These results clearly suggest that NF-κB activation and nuclear translocation occur in response to CGN-treatment in human colonocytes.

Our data also suggest that the mechanism of NF-κB activation in the intestinal cells following CGN exposure is largely attributable to increase in Bcl10. Although most studies of Bcl10 have been focused on lymphocytes and MALT (mucosal-associated lymphoid tissue) lymphomas (17), recent studies have indicated that Bcl10 may have more universal function in different types of tumors (14). Bcl10 normally resides in the cytoplasm to relay receptor-mediated signals to activate NF-κB (35). The pathway by which Bcl10 may activate NF-κB is not fully elucidated in our studies in intestinal cells. In lymphocytes, Bcl10 induced phosphorylation of IκB involves IKKγ
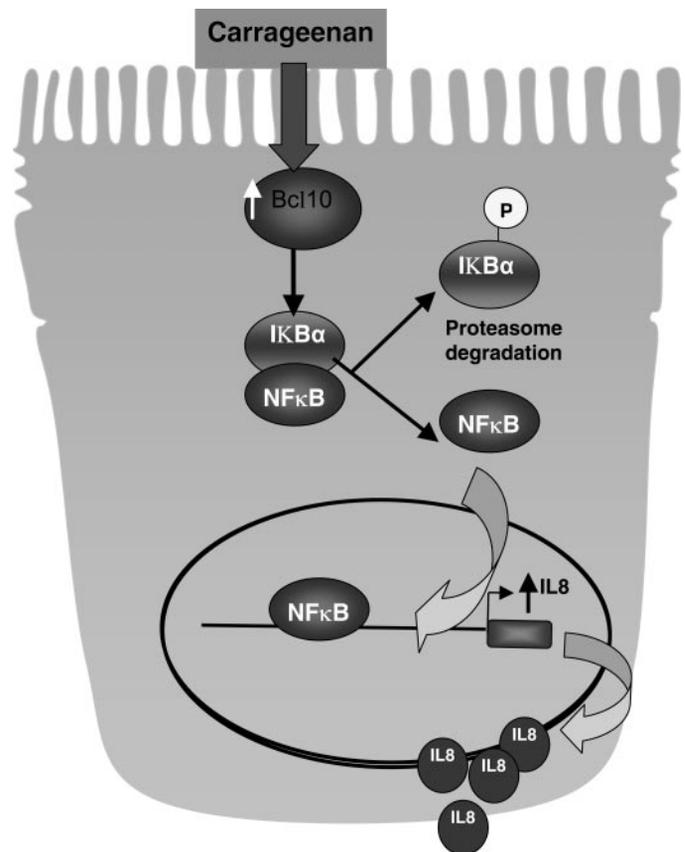

Fig. 8. Experimental model of IL-8 activation by λCGN in colonic epithelial cells. Drawing indicates *1*) activation of Bcl10 following CGN exposure, leading to *2*) phosphorylation of IκBα, *3*) activation and nuclear translocation of NF-κB, then *4*) transcriptional activation of IL-8 and increased IL-8 secretion.

(also identified as NEMO for NF-κB essential modifier). Bcl10 acts as an intermediate between upstream T- and B-cell receptors and NF-κB activation. Our studies have determined that knockdown of Bcl10 by siRNA significantly reduces the CGN-induced increases in phospho-IκBα and IL-8. Although our studies do not address the mechanism(s) and intermediates that form the association between Bcl10 and phosphorylated IκBα, concomitant elevation of both by CGN suggests that a pathway similar to that identified in lymphocytes may exist in the IEC.

A number of cell types produce IL-8 in response to various stimuli, including other cytokines, infectious agents such as viruses, and bacterial products like lipopolysaccharides (36). NF-κB plays a central role in transcriptional activation of the IL-8 gene in IEC (30). In the present study, exposure of NCM460 cells to λCGN for 24 and 48 h caused a twofold increase in IL-8 secretion, and parallel short-term experiments with fresh colonic tissues showed similar increases in IL-8 secretion in response to λCGN treatment. IL-8 promoter activity increased ~10-fold in the NCM460 cells compared with control and was inhibited by CAPE, suggesting that CGN-induced increase in IL-8 production involves transcriptional regulation mediated by NF-κB. In the presence of CAPE (29), CGN treatment failed to induce increased secretion of IL-8 in NCM460 cells. These results suggest that CGN-induced IL-8 production is mediated via the NF-κB pathway in the colonic cells. Although in other systems NF-κB has been shown to be the mediator of CGN-induced inflammatory response, we have demonstrated for the first time an NF-κB-dependent secretion of IL-8 in response to CGN exposure in colonic epithelial cells.

Previous reports have shown NF-κB dependent IL-8 production by IEC in response to enteropathogenic *E. coli* infection (36) and hyperosmotic stress (30). However, since we found no alteration in osmolarity due to CGN, our results suggest that CGN-induced inflammatory changes are not secondary to its effect on osmolarity. Since λCGN resembles to some extent the naturally occurring glycosaminoglycans, but differs owing to its unusual α-1,3-glycosidic bond between sulfated galactose residues, we suspect that CGN may trigger Bcl10 activation by an immunological mechanism. Knockdown of Bcl10 demonstrated the significant role of Bcl10 in CGN-induced IL-8 secretion in the NCM460 cells. Since Bcl10 is a CARD-containing protein, the resemblance between this CGN-activated pathway via Bcl10 and the known genetic predisposition to Crohn's disease associated with mutations in NOD2/CARD15 is intriguing (15, 31) and suggests a possible intersection between genetic and environmental sources of intestinal inflammation at the CARD locus. In summary, the present study provides evidence that CGN stimulates an inflammatory cascade in normal colonic epithelial cells. This novel pathway involves Bcl10 activation, increase in phospho-IκBα, nuclear translocation of NF-κB, and increased production of IL-8 by a transcriptional mechanism. This proposed pathway of CGN-induced inflammation in normal human colonic epithelium may have important clinical implications, due to the widespread use of CGN in the human diet.

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