Activation of guanylate cyclase C signaling pathway protects intestinal epithelial cells from acute radiation-induced apoptosis


Division of Gastroenterology, Hepatology, and Nutrition, Cincinnati Children’s Hospital Medical Center, Cincinnati, and the University of Cincinnati, Cincinnati, Ohio

Submitted 31 March 2008; accepted in final form 7 February 2009

Garin-Laflam MP, Steinbrecher KA, Rudolph JA, Mao J, Cohen MB. Activation of guanylate cyclase C signaling pathway protects intestinal epithelial cells from acute radiation-induced apoptosis. Am J Physiol Gastrointest Liver Physiol 296: G740–G749, 2009. First published February 12, 2009; doi:10.1152/ajpgi.90268.2008.—Uroguanylin (UGN) is a peptide hormone that binds to and activates the intestinal epithelial cell (IEC) transmembrane receptor guanylate cyclase C (GC-C), which in turn increases intracellular cGMP. Gene targeting of murine UGN or GC-C results in significantly lower levels of cGMP in IECs. On the basis of effects of cGMP in nonintestinal systems, we hypothesized that loss of GC-C activation would increase intestinal epithelial apoptosis following radiation-induced injury. We first compared apoptosis from the proximal jejunum of C57BL/6 wild-type (WT) and GC-C knockout (KO) mice 3 h after they received 5 Gy of γ-irradiation. We then investigated whether supplementation via intraperitoneal injection of 1 mM 8BrcGMP would mitigate radiation-induced apoptosis in these experimental animals. Identical experiments were performed in BALB/c UGN WT and KO mice. Apoptosis was assessed by quantitating morphological indications of cell death, terminal dUTP nick-end labeling, and cleaved caspase 3 immunohistochemistry. Both UGN KO and GC-C KO mice were more susceptible than their WT littermates in this in vivo model of apoptotic injury. Furthermore, cGMP supplementation in both GC-C and UGN KO animals ameliorated radiation-induced apoptosis. Neither WT strain demonstrated significant alteration in apoptotic susceptibility as a result of cGMP supplementation before radiation injury. These in vivo findings demonstrate increased radiosensitivity of IECs in UGN and GC-C KO mice and a role for cGMP as a primary downstream mediator of GC-C activation in the protection of these IECs from radiation-induced apoptosis.

Cyclic guanosine monophosphate (cGMP); cleaved caspase 3; intestinal injury; uroguanylin; terminal dUTP nick-end labeling

GUANYLATE CYCLASE C (GC-C) is one of a family of transmembrane enzyme-linked receptors found on the luminal surface of intestinal epithelial cells. Upon ligand binding, GC-C catalyzes the intracellular conversion of GTP to cGMP. GC-C has two known endogenous ligands, uroguanylin and guanylin, and is also activated by the bacterial peptide Escherichia coli heat-stable enterotoxin (STA). Studies in GC-C knockout (KO) animals have confirmed the role of this signaling pathway in pathological intestinal secretion mediated by STA (27). Although GC-C is critical for the fluid and electrolyte response to STA, this receptor may have additional functions in normal physiology that remain to be defined.

In addition to fluid secretion, GC-C has been implicated in intestinal cell turnover. The intestinal epithelium is unique for its high cell turnover rate in which epithelial proliferation, differentiation, and eventual shedding into the lumen occur within days. Programmed cell death, or apoptosis, is an important process for eliminating senescent, damaged, or deleterious cells. Increased or decreased intestinal epithelial apoptosis is believed to be involved in the pathophysiology of multiple disease states including sepsis, cancer, radiation injury, inflammatory bowel disease, and ischemia-reperfusion injury (3, 5, 16, 21, 30, 32). In particular, radiation injury has been well studied. In the normal intestinal epithelium, there exists a low level of apoptosis that is balanced by proliferation of undifferentiated cells within the crypt. This compartment is especially vulnerable to apoptosis. Clinically, radiation-induced gastrointestinal dysfunction can be manifested by bloating and abdominal pain as well as diarrhea, malabsorption, and increased susceptibility to infection (24, 4, 10).

Although GC-C has been implicated in intestinal epithelial cell (IEC) apoptosis, its precise role remains to be defined. In a single study, UGN activation of GC-C–induced apoptosis in T84 cells and reduced number of polyps found in the intestine of Apc(Min/+) mice (23). However, other investigations did not observe any change in apoptosis by terminal dUTP nick-end labeling (TUNEL) assay in either T84 or Caco2 cell lines (17). In vivo studies demonstrated that absence of GC-C activation led to more abundant apoptosis of colonocytes but not the small intestine of Apc(Min/+). GC-C KO animals (11). cGMP accumulation has been proposed to be antiapoptotic in nonintestinal in vitro models as well. Studies have examined the role of soluble guanylate cyclase (sGC) in human ovarian and lymphocytic leukemia, as well as immortalized uterine cell lines (2, 6, 7). With these experiments either the addition of sGC decreased apoptosis or inhibitors of GC activity increased apoptosis. Furthermore, in a uterine cell line, supplementation of 8BrcGMP decreased the effect of GC inhibitors, suggesting that normal levels of cGMP were key to regulating apoptosis (2).

Previous studies of normal healthy nonirradiated mice show a steady low level of apoptosis that occurs with a frequency of 0.1–0.2 apoptotic events per sectioned crypt (20). Radiation is a well-established model for examining the induction of in vivo intestinal epithelial apoptosis (18). The present study addresses the role of GC-C and cGMP in regulating IEC apoptosis in mouse proximal intestine after radiation-induced injury. On the basis of our data we suggest that physiological GC-C activation reduces the occurrence of radiation-induced apoptosis in small IECs and diminished levels of cGMP in IECs result in increased sensitivity to radiation-induced apoptosis.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: M. B. Cohen, MLC 2010, 3333 Burnet Ave., Cincinnati, OH 45229-3039 (e-mail: mitchell.cohen@chmc.org).
MATERIALS AND METHODS

Animals

Age-matched adult mice (average weight 25 g) were used for the experiments. Since no differences were observed between males and females, data from animals of both sexes were pooled. GC-C null animals were constructed as previously described and inbred at least five generations into C57BL/6 (12, 14). The UGN KO and WT experiments were conducted on a BALB/c background. All mice were studied under a protocol approved by the Cincinnati Children’s Hospital Medical Center Institutional Animal Care and Use Committee. Mice had access to food and water ad libitum and were housed in a temperature-, humidity-, and light cycle-controlled, specific pathogen-free microisolation facility.

Exposure of Animals to γ-Radiation and Tissue Harvest

In the experimental group, 5 Gy total body γ-irradiation was delivered by a self-contained Cesium137 irradiator (Mark I-68) at a rate of 55 Gy/min (J. L. Shepherd & Associates, San Fernando, CA). We demonstrated that this was the lowest dose that produced maximal radiation-induced IEC apoptosis (data not shown). To minimize differences in circadian expression of uroguanylin, experiments were performed during a daily 2- to 3-h window (19). At 3 h after exposure, the proximal jejunum was collected by removing the proximal third of the small intestine after exclusion of duodenum and flushed with ice-cold 1× PBS. After longitudinal sectioning, two representative 1-cm pieces of tissue were collected and fixed flat in 10% neutral-buffered formalin. After standard processing and embedding, 5-μm sections were stained with hematoxylin and eosin (H&E) for light microscopic examination.

Immunohistochemistry

TUNEL. Tissue sections were deparaffinized, rehydrated, and digested with Proteinase K (DAKO, Carpinteria, CA). After 10 min blocking of endogenous peroxidase with 3% hydrogen peroxide, sections were incubated with reaction buffer containing biotin-labeled dUTP and freshly prepared terminal transferase solution at room temperature for 2 h (Roche Diagnostics, Indianapolis, IN). Sections were then incubated with Vectastain ABC-alkaline phosphatase kit (Vector Laboratories, Burlingame, CA) according to manufacturer’s instructions. Staining was detected with Sigma Fast Red (Sigma-Aldrich, St. Louis, MO).

CC3. After slides were deparaffinized and rehydrated, tissue sections underwent antigen retrieval by use of 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with a 1% hydrogen peroxide–methanol solution for 15 min. After being washed, samples were placed in a 0.2% Triton-X solution for 15 min to further enhance antigen exposure. After 1 h preincubation with normal goat serum, sections were incubated with 1:100 rabbit polyclonal anti-cleaved caspase 3 (CC3) antibody (Cell Signaling Technology, Danvers, MA) overnight at 4°C. After being washed, tissue samples were incubated with biotinylated goat anti-rabbit secondary antibody for 60 min, followed by development of avidin-biotin-peroxidase complex according to the manufacturer’s recommended instructions. Staining was detected by 3,3′-diaminobenzidine as the substrate (Vector Laboratories). Sections were counterstained with Harris hematoxylin (Fisher Scientific, Pittsburgh, PA). As a negative control, primary antibody was omitted.

Effect of cGMP Analogs on Apoptosis

To assess the effect of CGMP supplementation on apoptosis, cGMP was administered by 100 μl intraperitoneal (IP) injection of 1 mM 8BrcGMP (Sigma-Aldrich), a partially membrane-soluble cGMP analog, in PBS 1 h prior to irradiation (1.8 mg/kg). Control and UGN or GC-C KO mice were injected in a similar fashion with PBS alone. The proximal jejunum was dissected, flushed with cold PBS, and laid flat as described above. A glass slide was then used to scrape 2 cm of the intestinal mucosal surface from each mouse harvested. The scrapings were immediately placed in liquid nitrogen and subsequently stored at −80°C. On the day of analysis, tissue was homogenized in 500 μl of 0.1 M HCl. CGMP was then measured with a cGMP ELISA kit (Assay Designs, Ann Arbor, MI), per manufacturer’s instructions. Subsequent dosing experiments were performed in a similar fashion utilizing 8BrcGMP, 0.1 to 5 mM (0.18 to 9 mg/kg).

To test the effect of cGMP inhibition on apoptosis, a dosing experiment was performed with 100 μM of 0.01 to 2.5 mM Rp-8-pCPT-cGMPS, a cell-permeable cGMP antagonist/protein kinase G inhibitor (0.021 to 5.3 mg/kg). This cGMP analog was purchased from Biomol International (Plymouth Meeting, PA).

Quantification of Intestinal Apoptosis

For H&E-stained slides, the number of apoptotic cells per crypt were assessed by morphological criteria (25). Immunohistochemistry (IHC) was scored by assessing all cells positively stained via the TUNEL assay. IHC was additionally scored by recording all cells staining positive with use of the anti-CC3 antibody. Well-oriented crypts (20 crypts/animal), defined as sections containing Paneth cells, a crypt lumen, and an uninterrupted column of epithelial cells extending to the crypt-villus junction, were used for scoring. All scores were tabulated in a blinded fashion (M. P. Garin-Laflam).

RNA Isolation and cDNA Preparation

Total RNA was extracted from proximal jejunum with the RNeasy kit (Qiagen, Valencia, CA). After RNA preparation, samples were treated with DNase I to remove residual genomic DNA. Total RNA samples (2 μg) were reverse transcribed into cDNA by using random hexamers (SuperScript II, Invitrogen, Carlsbad, CA).

Quantitative Real-Time PCR

All reactions were performed in triplicate with Brilliant SYBR Green QPCR Mix (Stratagene, La Jolla, CA) in the Stratagene Mx3000 system. PCR conditions were as follows: denaturing at 95°C, primer annealing at 55°C for 1 min, and primer extension at 72°C for 30 s. Primer sequences used were UGN forward 5′-TGAAGTTGGAG-GAGAAAGAGATGTG-3′ and reverse 5′-AAGGGCAAGGCT-GGTTATG-3′. Negative control was included in each run in which the template was replaced by an equal volume of water. Amplification of GAPDH mRNA was used as the loading control. The absolute levels of the mRNA were normalized with respect to GAPDH mRNA content. Amplification efficiencies ranged from 92 to 110%. Absence of primer-dimers was confirmed by postamplification by melting curve analysis.

Tissue Lysates and Western Blotting

Whole proximal jejunal tissue (5 cm) was harvested as detailed above. Tissue was homogenized in 500 μl RIPA buffer containing 0.5% (wt/vol) sodium deoxycholate, 10% Nonidet P-40, and 1% SDS diluted in PBS. Before lysis, 1 mM sodium orthovanadate, 50 μM NaF, 50 μM PMSF, and 1× Roche Complete protease inhibitor mix were added to RIPA buffer. Lysates were centrifuged at 15,000 rpm for 30 min. Protein samples were separated with NuPAGE Bis-Tris Gels (Invitrogen) and transferred to nitrocellulose membranes. Immunoblots were normalized by using equal amounts of protein (60 μg) as quantified by Bradford assay (Bio-Rad, Hercules, CA). After transfer, equal protein was confirmed by Ponceau S staining. Protein expression was additionally normalized to GAPDH expression, with the exception of radiated murine tissues. With the latter tissues, equal loading was confirmed by Coomassie staining and images were taken for reference. Membranes were blocked for 1 h in Tris-buffered saline.
with 0.1% Tween 20 and 1% bovine serum albumin, incubated with primary antibody overnight at 4°C, and subsequently incubated with horseradish peroxidase secondary antibody for 1 h. Anti-Bax (1:2,500) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-Bcl-w (1:500) was purchased from Axxora Life Sciences (San Diego, CA). Anti-soluble guanylyl cyclase (1:500) was purchased from Cayman Chemicals (Ann Arbor, MI). Anti-GAPDH (1:1,000) was purchased from Abcam (Cambridge, MA). Anti-pro-UGN antibody (1:1,000) was a gift from Michael Goy, University of North Carolina. Anti-GC-C antibody (1:500) was purchased from FabGennix International (Frisco, TX). Protein to probe for the GC-C receptor was enriched for the membrane portion of small intestinal tissue by using a previously published protocol (29). Immunoreactive signal was detected with the enhanced chemiluminescence method (Pierce Biotechnology, Rockford, IL). Densitometry of scanned images was performed with the ImageQuant software.

p53 ELISA

The p53 levels from WT and GC-C KO mice after γ-irradiation were evaluated by ELISA. After thorough flushing with cold PBS, 5 cm of proximal jejunal tissue was harvested. Samples were placed immediately in liquid nitrogen and stored at −80°C until further use. Proximal jejunal tissue from WT and GC-C KO mice not exposed to γ-irradiation were used as controls. The p53 ELISA kit (Duoset, DYC1355) was purchased from R&D Systems (Minneapolis, MN). Tissue samples were homogenized with extraction buffer (RIPA buffer as previously described). Samples were further sonicated and centrifuged, and the supernatant was aliquoted into fresh prelabeled tubes. One milligram of protein was loaded per well. Further details of ELISA protocol were performed per manufacturer’s instructions.

Statistical Analysis

All values are expressed as means ± SE. All comparisons unless otherwise indicated are made between WT and KO mice by the Student’s t-test. Comparisons of data among multiple groups were performed with one-way ANOVA followed by the unpaired Student’s t-test. Differences are considered significant at P < 0.05.

RESULTS

Radiation-Induced Apoptosis Is Increased in GC-C KO Mice

Figure 1A illustrates spontaneous apoptosis in both WT and GC-C KO mice and levels of apoptosis in both genotypes 3 h after γ-irradiation by H&E analysis. At baseline there was slightly less than one apoptotic event per every six to nine crypts. Exposure to γ-irradiation increased IEC apoptosis within crypt cells by >10-fold from nonirradiated tissue in

![Fig. 1. Effect of γ-irradiation on crypt cell apoptosis in wild-type (WT) and guanylate cyclase C (GC-C) knockout (KO) C57BL/6 mice. Shown are means ± SE of apoptotic events per crypt in the proximal jejunum of WT (solid bars) and GC-C KO (open bars) of either baseline apoptosis (0 Gy) or after 3 h of 5 Gy γ-irradiation as assessed by hematoxylin and eosin (H&E) analysis (n = 6–11 animals/group), *P < 0.01 (A), terminal dUTP nick-end labeling (TUNEL) staining (n = 4–7 animals/group), *P < 0.01 (C), and cleaved caspase 3 staining (n = 6–8 animals/group), **P = 0.05 (E). Representative nonradiated and radiated WT and GC-C KO crypts depicting apoptosis by apoptotic bodies indicated with white arrows (B), TUNEL staining in red (D), and cleaved caspase 3 staining in brown (F).](http://ajpgi.physiology.org/)

AJP-Gastrointest Liver Physiol • VOL 296 • APRIL 2009 • www.ajpgi.org
both WT and GC-C KO animals. However, radiation injury resulted in >90% more apoptotic cells in GC-C KO mice compared with WT controls. Pictorial representations of apoptotic bodies by H&E analysis are shown in Fig. 1B. Spontaneous apoptosis was also evaluated by TUNEL and CC3 in both WT and GC-C KO mice (Fig. 1, C and E). With both modalities of analysis, no statistical differences in apoptotic events were noted between genotypes at baseline and both genotypes had >10-fold increase from baseline apoptosis. Again, GC-C KO animals had an increase in the number of apoptotic events per crypt compared with their WT littermates. TUNEL analysis more closely paralleled the morphological quantification of apoptosis with a >90% vs. a 17% increase with CC3 analysis. Representative sections demonstrating TUNEL and CC3 staining are shown in Fig. 1, D and F.

dGMP Supplementation Decreases Radiation-Induced Apoptosis in GC-C KO Mice

Previous studies demonstrated that GC-C KO mice have diminished activity of guanylyl cyclase compared with WT controls (22). As expected, we found that cGMP levels were lower in GC-C KO mice and confirmed that radiation did not significantly alter the relative cGMP levels between genotypes (Fig. 2A). Radiation did not significantly increase UGN mRNA expression in WT or GC-C KO animals (Fig. 2B). To test whether low cGMP levels could be the mechanism by which GC-C KO mice have increased apoptotic susceptibility to radiation, we attempted to rescue these mice with a synthetic cGMP analog. We first validated the approach to increasing tissue levels of cGMP. Both WT and GC-C KO animals received either a 1 mM (1.8 mg/kg) IP injection of the cGMP analog, 8BrcGMP, or a PBS vehicle control. At 60 min after injection, cGMP levels within the proximal jejunum were increased >20-fold compared with vehicle-treated animals (Fig. 2C). Absolute cGMP levels in WT mice were higher than GC-C KO animals despite equivalent IP injection.

To confirm that decreased cGMP levels in GC-C null mice result in elevated sensitivity to radiation injury, we next evaluated whether systemically administered cGMP would raise IEC cGMP levels sufficiently to be cytoprotective and rescue the KO phenotype (Fig. 3, A–B). After cGMP administration, the irradiation model was employed identically to the initial GC-C experiments. By H&E, GC-C KO mice supplemented with the cGMP analog had half the number of apoptotic bodies per crypt compared with vehicle injected GC-C KO mice (Fig. 3C). By TUNEL assay, GC-C KO mice receiving 8BrcGMP injection also had half the number of apoptotic events per crypt compared with vehicle-treated mice (Fig. 3D). As measured by CC3 IHC, supplemented GC-C KO mice had 63% fewer positively stained cells per crypt than GC-C KO mice receiving only the vehicle control (Fig. 3E).

The apoptotic events in GC-C KO animals receiving 8BrcGMP injection were decreased to levels similar to that of vehicle-treated WT mice exposed to radiation injury. However, pretreatment with 8BrcGMP before γ-irradiation in GC-C WT mice did not alter the apoptotic levels compared with WT vehicle-treated animals.

Radiation-Induced Apoptosis Is Increased in UGN KO Mice

To determine whether loss of the endogenous GC-C ligand, UGN, would mimic the results seen with loss of the GC-C receptor, we investigated UGN KO mice. UGN KO animals are completely devoid of UGN expression and have significantly diminished GN expression (12). Figure 4A illustrates the frequency of spontaneous apoptosis in WT and UGN KO mice and levels of apoptosis in both genotypes 3 h after irradiation by H&E analysis. No significant difference was appreciated between genotypes before irradiation. Similar to GC-C WT and KO mice, exposure to γ-irradiation increased IEC apoptosis within crypt cells by >10-fold from nonirradiated tissue in both WT and UGN KO animals. At 3 h after radiation injury there were 43% more apoptotic events in UGN KO mice compared with WT controls (Fig. 4, A and B).

Spontaneous apoptosis was also evaluated by TUNEL and CC3 analysis in both WT and UGN KO mice (Fig. 4, C and E). As observed with H&E staining, there was no significant

Fig. 2. Relative cGMP levels between WT and GC-C KO mice. A: levels of cGMP at baseline (0 Gy) were ~2.5-3-fold higher in the proximal jejunum in WT-type C57BL/6 compared with GC-C KO animals. These relative differences did not alter after exposure to 5 Gy γ-irradiation (n = 3–6 animals/group). *P < 0.05. B: RNA was extracted from WT and GC-C KO mice at either baseline (0 Gy) or 3 h after 5 Gy. Uroguanylin (UGN) expression by RT-PCR after radiation was not significantly elevated in either WT or KO animals (n = 3–6 animals/group). C: WT C57BL/6 mice treated with PBS vehicle for 60 min again had >3-fold higher cGMP levels compared with GC-C KO mice. After intraperitoneal injection of 8BrcGMP, total cGMP was significantly increased in both genotypes. However, cGMP levels in proximal jejunal tissue from WT animals were >80-fold higher than in GC-C KO mice. Comparing genotypes WT and KO mice individually, cGMP levels were increased >20-fold and >30-fold, respectively, from vehicle-treated (Veh) mice (n = 2–3 animals/group). **P < 0.01.
difference between genotypes. At 3 h after radiation exposure, apoptosis was increased by TUNEL and CC3 staining in both genotypes. TUNEL analysis demonstrates that UGN KO animals had 52% more positive staining cells than WT controls (Fig. 4, C and D). By CC3 IHC, UGN KO mice had an increase of 15% in the number of positive staining cells compared with the WT littermates (Fig. 4, E and F).

cGMP Supplementation in UGN KO Animals Decreased Radiation-Induced Apoptosis

In an experimental design identical to that used in GC-C KO and WT animals, we evaluated the cytoprotective role of cGMP supplementation prior to radiation exposure in UGN KO and WT mice. Figure 5A illustrates that similar to GC-C KO animals, UGN KO animals have lower cGMP levels than their WT littermates. Furthermore, γ-radiation does not significantly alter these relative differences.

WT mice supplemented with cGMP showed no significant differences in IEC apoptotic response evaluated by H&E or CC3 IHC to radiation-induced injury (not shown). By H&E analysis, 8BrcGMP supplementation resulted in a 31% decrease in UGN KO mice IEC apoptosis compared with vehicle-treated mice (Fig. 5B). When IEC apoptosis was quantified by use of CC3 IHC, there was a 21% decrease in apoptosis in the UGN KO mice treated with cGMP supplementation compared with the PBS-injected controls (Fig. 5B).

GC-C KO Mice Are More Sensitive to Amelioration of Radiation-Induced Apoptosis by cGMP Supplementation

To further define the effect of cGMP supplementation in this radiation model, we conducted an 8BrcGMP dosing experiment of WT and GC-C KO mice. In an experimental design identical to previous cGMP supplementation studies (Fig. 3B), WT and GC-C KO animals received either vehicle control or the following doses of 8BrcGMP: 0.1, 1, or 5 mM (0.18, 1.8, or 9 mg/kg). Apoptosis in these studies was quantified by morphological examination. In GC-C KO animals, amelioration of radiation-induced apoptosis to levels equivalent to WT littermates occurs even with the lowest dose tested. In contrast, decrease of apoptotic phenotype in WT mice requires the highest tested dosing of this semipermeable cGMP analog (Fig. 6A).

Administration of cGMP Antagonist, Rp-8-pCPT-cGMPS, Increases Radiation Apoptosis in both WT and GC-C KO Animals

To test the specificity of the therapeutic effect of cGMP supplementation, WT and GC-C KO animals received varying...
doses of Rp-8-pCPT-cGMPS, a cell-permeable cGMP antagonist, vs. vehicle control (1). At all doses tested, 0.01, 0.1, 1, and 2.5 mM (0.021, 0.21, 2.1, and 5.3 mg/kg), apoptosis in WT animals was significantly increased (Fig. 6A). Levels of apoptosis in GC-C KO animals were significantly increased at doses of 0.1 mM and 2.5 mM (Fig. 6A).

Molecular Analysis of Apoptotic Proteins in Small Intestinal Gamma Radiation Model

To further explore potential apoptotic pathways in this small intestinal γ-radiation model, two candidate proteins, bax and bcl-w, were investigated. We first confirmed by Western blot

![Image](http://ajpgi.physiology.org/)

**Fig. 4.** Effect of γ-irradiation on crypt cell apoptosis in WT and UGN KO BALB/c mice. A: mean ± SE of apoptotic events per crypt assessed by H&E in proximal jejunum of WT (solid bars) and UGN KO (open bars) of either baseline apoptosis (0 Gy) or after 3 h of 5 Gy γ-irradiation as quantified by H&E analysis (n = 6–10 animals/group), *P < 0.01 (A); TUNEL staining (n = 5–7 animals/group), *P < 0.01 (C); and cleaved caspase 3 staining (n = 6–10 animals/group), **P < 0.05 (E). Representative nonradiated and radiated WT and UGN KO crypts depicting apoptosis by apoptotic bodies indicated with white arrows (B), TUNEL staining in red (D), and cleaved caspase 3 staining in brown (F).

![Image](http://ajpgi.physiology.org/)

**Fig. 5.** 8BrcGMP attenuates apoptosis in small intestinal epithelium of UGN KO mice following γ-irradiation. A: levels of cGMP at baseline (0 Gy) were ~2.5–3-fold higher in the proximal jejunum in WT BALB/c compared with UGN KO animals (n = 3 animals/group), *P < 0.05. B: UGN KO BALB/c mice received an intraperitoneal injection of either vehicle (PBS) or 1 mM 8BrcGMP, followed by 5 Gy (n = 10–11 animals/group), **P < 0.01. Apoptotic events assessed by H&E (solid bars) vs. cleaved caspase 3 staining (open bars). With both modalities of quantifying apoptosis, cGMP supplementation decreased radiation-induced apoptosis compared with KO mice receiving PBS treatment alone.
that bax, a proapoptotic member of the bcl-2 family, was present in nonradiated murine GC-C KO tissue. Protein levels were then quantified in both WT and GC-C KO tissue from nonradiated and radiated jejunal tissue. Bax was expressed at similar levels at baseline in WT and KO animals (Fig. 6B). This expression did not significantly increase after 5 Gy γ-irradiation treatment in WT or KO animals (Fig. 6B).

Bcl-w, an antiapoptotic member of the bcl-2 family, was then quantitated. Protein levels by Western blot were evaluated from both WT and KO tissue, at baseline and in radiated animals. Although bcl-w protein expression is equally present at baseline (0 Gy), after γ-irradiation bcl-w expression is decreased in both WT and GC-C KO animals (Fig. 6C). This suggests that bcl-w is a target of radiation-induced injury.

p53 Levels Increase in Both WT and GC-C KO Animals in Response to γ-Radiation

To explore whether increased radiation-induced apoptosis in GC-C KO animals is p53 driven, we evaluated the change in p53 levels in both WT and GC-C KO animals after γ-irradiation. At baseline, p53 levels in GC-C KO jejunum were lower than WT animals. The fold change after radiation of p53 levels from baseline was 1.3 for WT and 1.8 for KO animals as shown in Fig. 6D.

Levels of sGC in GC-C and UGN KO Animals

To determine whether there was a compensatory increase in sGC in GC-C and UGN KO animals, we evaluated levels of sGC in jejunal tissue by Western blot analysis. No significant difference is appreciated in soluble GC levels between WT and GC-C KO mice (Fig. 7A). There is an increase in sGC expression in UGN KO mice compared with their WT littermates (Fig. 7B). Previously published articles have illustrated the complete loss of mRNA expression due to gene targeting in GC-C and UGN KO animals (12, 14). We have further confirmed by Western blot the absence of protein expression in these receptor and ligand KO animals as shown in Fig. 7, C and D, respectively.

Neutrophilic Infiltration Is Not a Variable in Acute, Nonlethal γ-Radiation Injury

To explore the potential mitigating factor of acute inflammation in low-dose radiation injury, we systematically quanti-
fied polymorphonuclear leukocytes (PMNs) by two approaches, 1) morphological assessment with light microscopy and 2) myeloperoxidase (MPO) staining. As anticipated, neutrophilic infiltration is not present at baseline in WT and GC-C KO mice. PMNs were similarly negligible in these genotypes 3 h after γ-radiation (data not shown). Absence of neutrophilic infiltration was then evaluated as a potential variable in UGN KO animals. We similarly confirmed by both H&E analysis and MPO IHC that neutrophils do not invade small intestinal crypt cells during baseline or radiation-induced apoptosis in either WT or UGN KO mice (data not shown).

**DISCUSSION**

The objective of the present study was to evaluate the role of GC-C activation in radiation-induced apoptosis. Our results suggest a cytoprotective function for the GC-C signaling pathway and are consistent with cGMP as a primary downstream mediator of this effect.

Our results are in contrast to the previous studies in which stimulation of the UGN-GC-C pathway led to either increased or unaltered apoptosis (17, 23). Our interest in exploring the relevance of GC-C signaling in apoptosis was driven by these conflicting earlier studies. We believe our present investigations address previous confounders. First and foremost, we have relied on in vivo rather than in vitro studies to best approximate the relevant physiological and pathophysiological interactions. Moreover, because previous studies have relied either on transformed cell lines or adenomatous in vivo models, in which there is dysregulated apoptosis, the effects of the GC-C signaling cascade may be altered.

In a recent study, there was more apoptosis in unperturbed GC-C KO mice than in WT mice (11). Although we did not see a statistical difference in our experiments at baseline, our findings of an antiapoptotic effect mediated by the UGN-GC-C pathway are consistent with this observation and are supported by two sets of experimental findings. First, both ligand and receptor KO animals have lower cGMP levels at baseline than their WT counterparts and both have increased susceptibility to radiation-induced apoptosis. That a similar effect is seen in both KO genotypes is not unexpected. Although UGN is not the only ligand for GC-C, we have previously shown that our UGN KO animals have lower cGMP levels at baseline than their WT counterparts and both have increased susceptibility to radiation-induced apoptosis. Although UGN-KO animals have extremely low levels of guanylin, the other major GC-C ligand. Thus it was not surprising to find that loss of both ligands (UGN and guanylin) mimicked the loss of the common receptor, GC-C. However, recent studies in the Rac/p21-activated kinase (PAK) signaling pathway have demonstrated that ligand activation of transmembrane guanylate cyclases is not the only mechanism of activation and resulting increase in intracellular cGMP levels. These investigations demonstrated that intracellular PAK can allosterically activate some guanylate cyclase receptors in the absence of their cognate ligands (8). Therefore, the similar phenotype we observed in both ligand and receptor KO suggests that ligand activation is required for GC-C to protect against apoptosis.
Our second observation was that cGMP supplementation ameliorated radiation-induced apoptosis in the GC-C KO mice. Furthermore, supplemented GC-C KO animals exposed to γ-irradiation now had equivalent apoptotic levels to irradiated WT controls. Experimental supplementation of UGN KO animals also mitigated radiation-induced apoptosis. Therefore, cGMP is likely part of the downstream signaling pathway that mediates resistance to apoptosis. A potential limitation of our approach is that the increase in cGMP was not targeted specifically to the IECs. Clearly, other cell types can be sensitive to the effects of cGMP. In fact, a precedent for involvement of cGMP in apoptosis is also supported by studies in rat myocytes, pancreatic β cells, and endothelial cells (13, 26, 31). Our initial data from both WT strains tested with 1 mM (1.8 mg/kg) 8BrcGMP did not demonstrate further protection against apoptosis due to radiation injury. Our subsequent 8BrcGMP dosing experiment of WT and GC-C KO mice revealed that WT mice require more cGMP supplementation to ameliorate radiation-induced apoptosis. Although this does not exclude a critical threshold, in which excess cGMP cannot mediate any further protective effect (at a higher concentration not yet tested), it does underscore that GC-C KO animals are more exquisitely sensitive to cGMP supplementation than their WT littermates.

Because there is no single, gold standard for evaluation of IEC apoptosis, we sought to employ three methods of quantification: morphological analysis by H&E, TUNEL, and CC3 IHC. H&E analysis and TUNEL assay were strongly correlated. Although generally lower trends were observed by CC3 IHC, the directional trends were similar with all three methods. The discrepancy between CC3 and the other two modalities of quantifying apoptosis has been documented before (28). Therefore, these results could be secondary to intrinsic limitations between these complementary assays. Because CC3 activity is early in the pathway of apoptosis, it is also possible that this experimental design selects for the later morphologically distinct stages of programmed cell death. Lastly, the lack of complete concordance between CC3 vs. H&E or TUNEL analysis could suggest that the mechanism of apoptosis in these KO animals is only partially caspase 3 dependent.

Our subsequent series of experiments were designed to further demonstrate the specificity of cGMP in modulating radiation-induced apoptosis. As our initial studies illustrated, GC-C KO mice, which are known to have decreased cGMP from WT animals, have increased apoptotic susceptibility to radiation injury. Furthermore, pharmacological supplementation with cGMP in these KO mice ameliorates this apoptosis. We next sought to evaluate whether pharmacological disruption of the action of cGMP would mirror the increased apoptotic susceptibility to radiation observed in these transgenic KO animals. The dosing experiment conducted in GC-C WT and KO mice utilized Rp-8-pCPT-cGMPS, which functions as a cGMP antagonist. Its direct action is to inhibit a downstream target of cGMP, protein kinase G. It is a cell-permeable analog of cGMP, which neither is hydrolyzed by nor stimulates cGMP phosphodiesterases and does not interact with cAMP-dependent protein kinases (1).

The results from this experiment demonstrate that administration of Rp-8-pCPT-cGMPS increased radiation-induced apoptosis in both WT and GC-C KO animals. Of note, the lowest doses did not further increase apoptosis in GC-C KO animals, in contrast to WT animals, in which we observed increased radiation-induced apoptosis with all doses administered. Nevertheless, the combined results of Rp-8-pCPT-cGMPS supplementation indicate that overall this cGMP analog was highly effective at increasing apoptotic susceptibility in both WT and GC-C KO animals. Not only does this dosing experiment consequently underscore the specificity of cGMP as a necessary modulator of this apoptotic injury pathway, but these findings also provide directional evidence that protein kinase G is a downstream mediator of radiation-induced apoptosis.

Another potential mechanism of apoptosis in these KO animals could be p53 activity. After exposure to low levels of radiation, IECs in the intestinal crypt are very susceptible to induction of apoptosis by a p53-dependent mechanism. Loss of p53 essentially renders IECs from the small intestine and the colon radiation resistant (15). In other nonintestinal cells susceptible to p53-dependent apoptosis, it has been shown that cell death is linked to guanylate cyclase activity. Fraser et al. (7) demonstrated in human ovarian cells that increased basal sGC activity reduced p53 and decreased p53-dependent apoptosis. These authors demonstrated that suppression of sGC activity with the specific inhibitor oxadialoquinoxalione (ODQ) increased apoptosis and that 8BrcGMP decreased apoptosis. In a different in vitro model of apoptosis, Heinloth et al. (9) observed that 8BrcGMP could decrease p53-dependent apoptosis of stimulated macrophages. This effect was similarly prevented by addition of ODQ, leading to both increased p53 stabilization and apoptosis.

Our present result of total p53 by ELISA falls short of allowing us to conclude that the GC-C signaling pathway in this model could be mitigating p53-dependent apoptosis. It is interesting to note that baseline GC-C KO levels of p53 were consistently lower than WT p53 levels. As a consequence, although the absolute p53 levels from GC-C KO mice were not higher than those of WT littermates, the fold change in KO animals was greater (1.8-fold change in GC-C KO vs. 1.3-fold change in WT C57 BL/6 mice). The physiological relevance of these differences remains unclear.

In conclusion, our in vivo findings demonstrate the increased apoptotic sensitivity of IECs in UGN and GC-C KO mice after radiation and provide evidence for the role of cGMP, as a primary downstream mediator of GC-C activation in the protection of these IECs from radiation-induced apoptosis. Our results differ from some previous experiments showing an apoptotic effect of UGN. It is certainly possible that the specific experimental conditions and environment may dictate the response to activation of GC-C. The possibility of a heterogeneous response to GC-C activation indicates the need for complete understanding of the downstream effects of activation before this ligand receptor system is used clinically to modulate either apoptotic injury or cell cycle regulation.

GRANTS

This work was supported by National Institutes of Health Grants T32 DK-07727, R01 DK-47318, T32 ES-10957, and F32 DK-80588.

REFERENCES

2. Chan SL, Fiscus RR. Guanylyl cyclase inhibitors NS2028 and ODQ and protein kinase G (PKG) inhibitor KTS823 trigger apoptotic DNA frag-


15. Pitari GM, Di Guglielmo MD, Park J, Schulz S, Waldman SA. Guanylyl cyclase C agonists regulate progression through the cell cycle of