Enterotoxin preconditioning restores calcium-sensing receptor-mediated cytostasis in colon cancer cells

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Guanylyl cyclase C (GCC), the receptor for diarrheagenic bacterial heat-stable enterotoxins (STs), inhibits colorectal cancer cell proliferation by co-opting Ca\(^{2+}\) as the intracellular messenger. Similarly, extracellular Ca\(^{2+}\) (Ca\(^{2+}\),o) opposes proliferation and induces terminal differentiation in intestinal epithelial cells. In that context, human colon cancer cells develop a phenotype characterized by insensitivity to cytostasis imposed by Ca\(^{2+}\),o. Here, preconditioning with ST, mediated by GCC signaling through cyclic nucleotide-gated channels, restored Ca\(^{2+}\),o-dependent cytostasis, reflecting posttranscriptional regulation of calcium-sensing receptors (CaRs). ST-induced GCC signaling deployed CaRs to the surface of human colon cancer cells, whereas elimination of GCC signaling in mice nearly abolished CaR expression in enterocytes. Moreover, ST-induced Ca\(^{2+}\),o-dependent cytostasis was abrogated by CaR-specific antisense oligonucleotides. Importantly, following ST preconditioning, newly expressed CaRs at the cell surface represented tumor cell receptor targets for antiproliferative signaling by CaR agonists. Since expression of the endogenous paracrine hormones for GCC is uniformly lost early in carcinogenesis, these observations offer a mechanistic explanation for the Ca\(^{2+}\),o-resistant phenotype of colon cancer cells. Restoration of antitumorigenic CaR signaling by GCC ligand replacement therapy represents a previously unrecognized paradigm for the prevention and treatment of human colorectal cancer employing dietary Ca\(^{2+}\) supplementation.

Introduction

Colorectal cancer is most prevalent in the developed world, representing the second leading cause of cancer-related mortality (1.2). Although the epidemiology of this disease is poorly understood, there is an unexplained inverse relationship between the worldwide incidence of colorectal cancer and enterotoxigenic Escherichia coli infections (3.4). Enterotoxigenic E. coli produces heat-stable enterotoxins (STs), exogenous ligands for the intestine-specific receptor guanylyl cyclase C (GCC) (5) and a principle cause of secretory diarrhoea in endemic populations and animal herds (6–8). Regions of the world with the highest incidence of enterotoxigenic E. coli-associated diarrhoea exhibit the lowest incidence of colon cancer (4). In that context, activation of GCC inhibits human colon carcinoma cell proliferation (4,9,10) and adenoa formation in mice (3). Reduced expression of the endogenous paracrine hormones for GCC, guanylin and uroguanylin, represents an early mutational event in colorectal carcinogenesis (11–13). GCC signaling through its second messenger cGMP promotes fluid and electrolyte secretion (5), opposes cell cycle progression and proliferation (4,9) and regulates migration, differentiation and apoptosis along the crypt–villus axis (14,15). Importantly, targeted GCC deletion (GCC\(^{−/−}\)) in mice increased intestinal tumorigenesis induced by the carcinogen azoxymethane or adenomatous polyposis coli (APC) mutations by corrupting homeostatic crypt proliferation and genomic integrity (16). These observations suggest a model in which colorectal cancer is a disease of hormone insufficiency where dysregulation of GCC signaling, following loss of guanylin and uroguanylin, promotes tumorigenesis by disrupting mucosal homeostasis (16). However, beyond production of cGMP, molecular mechanisms by which GCC regulates processes underlying carcinogenesis remain undefined.

Like GCC, extracellular Ca\(^{2+}\) (Ca\(^{2+}\),o) opposes proliferation and promotes differentiation of intestinal mucosal cells (17–20). Moreover, Ca\(^{2+}\) supplementation abrogates intestinal hyperproliferation and tumor formation induced by a western-style diet in APC\(^{Min/+}\) mice (21). In part, these antitumorigenic effects may reflect GCC-induced activation of cyclic nucleotide-gated (CNG) channels, inducing cytostasis through Ca\(^{2+}\),o influx (4). Notably, Ca\(^{2+}\),o in the colonic lumen may increase to \(\geq20\) mM that activates calcium-sensing receptors (CaRs) (19,22), G protein-coupled receptors (23) expressed in apical membranes of colonocytes (24), an event resulting in reduced proliferation and tumorigenesis through inhibition of \(β\)-catenin/Tcf-4 signaling, and increased cell maturation through p21 and p27 activities (25,26). Conversely, Ca\(^{2+}\),o supports colorectal cancer cell proliferation by capacitative entry through store-operated Ca\(^{2+}\) channels (27,28), which opposes antiproliferative GCC signaling through CNG and Ca\(^{2+}\),o entry (28). Thus, opposing mechanisms regulating proliferative balance by Ca\(^{2+}\),o comprise functional units reciprocally orchestrated in colon cancer cells (28).

Here, the functional relationship between GCC and Ca\(^{2+}\),o was explored in human colon carcinoma cells preconditioned with ST, in the context of a dynamic range of Ca\(^{2+}\),o and in GCC\(^{−/−}\) mice. These studies revealed a previously unappreciated role for GCC in regulating CaR signaling, offering a novel paradigm for the prevention and treatment of human colorectal cancer employing hormone replacement therapy with GCC ligands in combination with oral Ca\(^{2+}\) supplementation.

Materials and methods

Tumor cell proliferation

Proliferation of cancer cells was quantified in 96 wells per plate by [methyl-\(^{3}H\)]thymidine (0.2 \(\mu\)Ci/well) incorporation into DNA (9). Cells were pulse labeled (3 h) with \(^{3}H\)-thymidine at the end of 24 h periods of proliferation induced by 10 mM L-glutamine. Following \(^{3}H\)-thymidine labeling, media was aspirated, cells were incubated for 15 min with ice-cold 10% trichloroacetic acid and rinsed sequentially with 10% trichloroacetic acid and 100% methanol. The acid-insoluble material containing \(^{3}H\)-labeled DNA was solubilized in 100 \(\mu\)l of 0.2 N NaOH, 80 \(\mu\)l aliquots were dissolved in 1 ml ScintiVerse and radioactivity quantified in a Packard \(β\)-scintillation spectrometer. All experiments were conducted on exponentially growing tumor cells.

Tumor cell toxicity

Cytotoxicity, including occurrence of apoptosis or necrosis, was assessed by flow cytometry (9). Cancer cells (~10\(^{5}\) cells per well in six-well plates) were treated (24 h) with the indicated reagents. Then, cells were placed in suspension by trypsination, pelleted by centrifugation, washed with phosphate-buffered saline and fixed in ice-cold 75% ethanol (30 min). After another wash with phosphate-buffered saline, cells were resuspended in the staining solution (50 \(\mu\)g/ml propidium iodide, 100 \(\mu\)g/ml RNase A, 1 mM ethylendiaminetetraacetic acid and 0.1% Triton X-100) and analyzed on a Coulter EPICS XL-MCL flow cytometer. Distribution in different phases of the cell cycle was analyzed using WinMDI software (version 2.8) provided by Joseph Trotter, Scripps Research Institute (La Jolla, CA). Twenty thousand cells, cleared from doublets, were analyzed from each sample.

Cyclic GMP assay

GCC-induced intracellular cGMP accumulation was assessed after treating cancer cells in triplicate with ST (15 min in six well per plate) employing

Abbreviations: APC, adenomatous polyposis coli; Ca\(^{2+}\),o, extracellular Ca\(^{2+}\); CaR, calcium-sensing receptor; CNG, cyclic nucleotide-gated; GCC, guanylyl cyclase C; ST, heat-stable enterotoxin.

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Eagle’s minimal essential medium supplemented with 2 mM L-glutamine. Reactions were terminated by adding ice-cold 100% ethanol, each well was washed twice with ice-cold 100% ethanol and supernates separated from pellets by centrifugation (12,000g, 15 min at 4°C). Supernates containing cGMP were evaporated in a Savant SVC-100H concentrator (Thermo Electron Corporation, Waltham, MA) and resuspended with 50 mM sodium acetate (pH 4.0), and cGMP was quantified in each sample in triplicate by radioimmunoassay (4).

**Immunoblot analysis**

Proteins from total cell lysates, cytosol or membrane extracts (28) prepared in sodium dodecyl sulfate sample buffer were separated by electrophoresis on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred on nitrocellulose membranes and then probed with rabbit polyclonal antibodies directed against CaR (dilution 1:1000) or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000) or goat anti-villin antibody [Villin(C-19); 1:1000] in Tris-buffered saline–TWEEN (5% milk) overnight at 4°C. Then, membranes were probed with horseradish peroxidase-conjugated secondary antibody (dilution 1:5000) for 1 h at room temperature, and specific bands were visualized employing West Pico Chemiluminescent Substrate and subjected to densitometry. Immunoblots, performed under reducing conditions, exhibited only one specific band of ~125 kDa corresponding to the monomer form of CaR.

**Immunostaining analyses**

For immunohistochemistry, tumor cells were washed twice with cold phosphate-buffered saline and immediately fixed (30 min in 4% paraformaldehyde) at room temperature followed by quenching with 3% H2O2. CaR or CD104 at the cell surface was visualized employing (overnight at 4°C) rabbit anti-CaR (1:100) or mouse monoclonal anti-human CD104 (1:100), respectively, and the Histostain-plus kit. For immunofluorescence, GCC--/- mice, generated by neomycin-resistant gene insertion on an I-129 background (29) and backcrossed with a C57BL/6 strain for seven or more generations, were employed. All animals were treated in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and under a protocol approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. GCC+/+ and GCC--/- littermates (males 2 months old; N, three per genotype) were killed, the intestine collected, divided into anatomically comparable segments and processed for paraffin-based archival. Paraffin-embedded tissue sections (5 μm) were dehydrated, heated (two times for 5 min in citrate buffer, pH 6) for antigen retrieval, incubated overnight (4°C) with rabbit anti-CaR (1:100) or goat anti-villin (1:50) followed by incubation for 30 min (room temperature) with Alexa fluor 555 anti-rabbit IgG (for CaR) or Alexa fluor 488 anti-goat IgG (for villin). Digital images were acquired by computers attached to a light microscope or a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

**Cell transductions**

A 5’ 327 bp CaR fragment containing 8 bp of upstream DNA was cloned into MSCV-puro retroviral vectors in both sense and antisense orientations (30), confirmed by DNA sequencing. Antisense, sense and empty vectors were transfected along with the packaging vector pCL Ampho into HEK 293 cells (for 72 h). Then, tumor cells stably transfected along with the packaging vector pCL Ampho into HEK 293 cells employing Fugene. Viral supernatants harvested at 48 and 72 h after transfection were used to transduce T84 cells (for 72 h). Then, tumor cells stably transfected along with the packaging vector pCL Ampho into HEK 293 cells employing Fugene. Viral supernatants harvested at 48 and 72 h after transduction did not reflect enhanced GCC-dependent cytostasis by Ca 2+ o (Figure 1C2). Indeed, cytostasis induced by acute ST exposure was not altered by Ca 2+ o over a range of 1–50 mM (Figure 1C2), suggesting that the antiproliferative effects of ST and Ca 2+ o are mediated by distinct, non-interfering mechanisms. In contrast, ST preconditioning (24 h), which induces desensitization to GCC-dependent cytostasis in colon cancer cells (10), potentiates the inhibition of proliferation by Ca 2+ o (Figure 1C1). Thus, ST synergistically increased the potency (IC50: Ca 2+ o alone, 9.91 ± 1.09 mM; ST plus Ca 2+ o, 6.49 ± 1.13 mM) and efficacy (mean percentage of proliferation: Ca 2+ o alone, 61.30 ± 1.84; ST plus Ca 2+ o, 49.47 ± 2.14) of Ca 2+ o to inhibit colon cancer cell proliferation (P < 0.001; Figure 1C1). Importantly, while failing to inhibit cell proliferation, reflecting desensitization to cGMP signaling (10) (see conditions at 1 mM Ca 2+ o, in Figure 1C1), ST preconditioning revealed a novel antiproliferative mechanism that required ≥3 mM Ca 2+ o (Figure 1C2).

Potentiation of Ca 2+ o-induced cytostasis by ST preconditioning was mediated by GCC since ST, but not an inactive analog, inhibited (IC50, 62.22 ± 3.21 nM; Figure 2A) proliferation of human colon carcinoma cells expressing GCC, but not of cells lacking GCC (32) (Figure 2B). Similarly, the effects of ST preconditioning were greater in T84 cells, which exhibits the highest GCC expression (32), compared with Caco-2 cells (Figure 2B). Further, two membrane-permeant cGMP analogs mimicked (Figure 2B), whereas an inhibitor of cyclic nucleotide-hydrolyzing phosphodiesterases enhanced (Figure 2C), the effects of ST preconditioning on tumor cell proliferation. Conversely, two inhibitors of CNG channels and an intracellular Ca 2+ chelator, but not inhibitors of cGMP- or cAMP-dependent protein kinase, blocked ST-mediated potentiation of Ca 2+ o-dependent cytostasis (Figure 2C). Notably, ST did not induce cytotoxicity in colon cancer cells exposed to high Ca 2+ o (Figure 2D). Rather, ST delayed the progression of these tumor cells through the cell cycle (Figure 2D), consistent with the notion that GCC agonists are cytostatic agents for colon cancer (4,9,10). Together, these observations suggest that in colon cancer cells induction of Ca 2+ o-dependent cytostasis by ST preconditioning is mediated by cGMP-dependent activation of CNG channels and Ca 2+ o entry, the same effector mechanism mediating GCC-induced cell cycle delay (4).

**GCC regulates the function of CaRs in normal and malignant colonocytes**

Increased antiproliferative effects of Ca 2+ o following ST preconditioning did not reflect enhanced GCC-dependent cytostasis by Ca 2+ o, because increased Ca 2+ o did not enhance cytostasis induced by GCC signaling (+/−ST for 3 h in Figure 1C2) and did not prevent GCC-mediated desensitization in cGMP signaling (10) (Figure 3A). Rather, ST preconditioning enhanced Ca 2+ o-dependent cytostasis by coupling increased Ca 2+ o with intracellular signaling mechanisms induced by cGMP signaling (Figure 2A–C). Ca 2+ o induces cytostasis in colonocytes, in part, by activating CaR signaling (22), a mechanism typically lost during neoplastic transformation (26,33). ST preconditioning (3 h) augmented the complement of total cellular (supplementary Figure 1A is available at Carcinogenesis Online) and membrane-bound CaRs (Figure 3B) without significantly altering cytosolic CaRs (Figure 3B) or CaR messenger RNA levels (data not shown). Further, examination of specific cell fractions (supplementary Figure 1B is available at Carcinogenesis Online) revealed that ST treatment (3 h) induces ~80-fold increase of CaR at tumor cell membranes (supplementary Figure 1C is available at Carcinogenesis Online), reflecting translocation of ~60% of the total complement of CaR protein in cancer cells from the cytosol to the membrane compartment (supplementary Figure 1D is available at Carcinogenesis Online). Importantly, GCC signal deprivation, either in ligand-free tumor cells (Figure 4, in vitro panels on the left showing CaR surface staining
of T84 cells) or in enterocytes of GCC−/− mice (Figure 4, in vivo panels on the right), reduced CaRs in cell membranes. Induction of GCC signaling with bacterial enterotoxin in tumor cells or endogenous paracrine hormonal circuitry in enterocytes of GCC−/+/ mice, in turn, increased the complement of CaRs at the cell surface (Figure 4). Effects of ST preconditioning on CaR staining at the surface of cancer cells were selective (supplementary Figure 2 is available at Carcinogenesis Online), independent of elevated Ca2+, but dependent upon cGMP signaling through CNG (Figure 4). Moreover, compared with GCC−/+/ mice, CaR expression in brush-border membranes of GCC−/− enterocytes was uniformly attenuated along the entire rostrocaudal axis of the intestine in a GCC-specific fashion since the expression of a marker for absorptive cells, villin, remained unchanged in the same anatomical locations (Figure 4, in vivo panels showing representative sections from the jejunum). Thus, GCC signaling physiologically regulates posttranscriptional expression of CaRs in enterocytes, and ST preconditioning induces cell surface translocation of CaR receptors in human colon cancer cells.

**Discussion**

Enterotoxins STs (18 amino acid long) and endogenous paracrine hormones guanylin and uroguanylin (15/16 amino acid long) are structurally homologous peptides (35,36), which specifically bind to and activate GCC at enterocyte brush-border membranes inducing cGMP-dependent protein kinase-dependent ion channel currents (36). In this way, endogenous hormones regulate body fluid homeostasis, whereas STs, principle diarrheagenic agents maximally activating that pathway, permit enterotoxigenic bacteria to propagate in the environment (7,36–38). Of significance, GCC−/− mice exhibit increased proliferation, migration and apoptosis and decreased
differentiation along the crypt–villus axis (14) and are more susceptible to intestinal tumorigenesis induced by azoxymethane or APC mutations (16). Moreover, expression of guanylin and uroguanylin is uniformly lost early in intestinal carcinogenesis (11–13), loss of guanylin expression resulted in crypt hyperproliferation (15) and administration of GCC ligands inhibits intestinal tumor formation in APCMin/+ mice (3) and cell cycle progression of colon cancer cells (9). Thus beyond fluid homeostasis, GCC controls proliferation and tumorigenesis in intestine.

Mechanisms mediating antiproliferation by GCC appear diverse. GCC signaling through unidentified effectors engages colonic cell quality control mechanisms assuring genomic integrity and tumor suppression (16). Also, GCC- and cGMP-dependent Ca²⁺ influx through CNGs induces colon cancer cell cytostasis by reducing differentiation along the crypt–villus axis (14) and are more susceptible to intestinal tumorigenesis induced by azaomethane or APC mutations (16). Moreover, expression of guanylin and uroguanylin is uniformly lost early in intestinal carcinogenesis (11–13), loss of guanylin expression resulted in crypt hyperproliferation (15) and administration of GCC ligands inhibits intestinal tumor formation in APCMin/+ mice (3) and cell cycle progression of colon cancer cells (9). Thus beyond fluid homeostasis, GCC controls proliferation and tumorigenesis in intestine.

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Fig. 5. CaR induced by ST is a novel therapeutic target in human colon cancer cells. (A and B) The proliferation of T84 cells stably expressing the empty vector (vector, control), the CaR antisense vector (AS-CaR) or the CaR sense vector (S-CaR) was examined. Cells were treated for 24 h with Ca2+o (5 mM) alone (A) or ST (1 μM) plus Ca2+o (5 mM) (B). Results are expressed as in Figure 1C2; the controls were low (0.5 mM; A) or high (5 mM; B) Ca2+o. *P < 0.05 versus parallel control conditions. (C) T84 cell proliferation exposed to CaR agonists was measured as the percentage of respective control incubations treated with 0.5 mM Ca2+o (first two columns on the left). CaR agonists were added to tumor incubations for 24 h in the presence of the vehicle control (PBS) or ST (1 μM), and include Ca2+o, 10 mM; Mg2+o, 20 mM; Gd3+o, 100 μM; spermine, 100 μM. CaR agonists are used at concentrations that maximally induce CaR signaling (34,40). *P < 0.05 and **P < 0.01 versus respective control [phosphate-buffered saline (PBS)] conditions. (D) Proposed molecular mechanism for restoration of CaR signaling by GCC associated with colorectal cancer suppression (see Discussion for description).
nuclear rates of DNA synthesis (4,9). Here, a previously unappreciated mechanism contributing to tumor suppression by GCC is described (Figure 5D). ST preconditioning induces cGMP-mediated CNG signaling and synthesis and cell surface delivery of CaRs. Increased CaRs at the plasma membrane, in turn, provide new tumor-specific targets for antiproliferative signaling by CaR agonists, including Ca\(^{2+}\)o, Mg\(^{2+}\), Gd\(^{3+}\), and spermine. Although mediated by the same proximal effector, Ca\(^{2+}\)o influx through CNG, inhibition of DNA synthesis (4) and activation of CaR signaling (Figure 5D) represent two distinct, non-overlapping GCC-dependent antiproliferative mechanisms, distinguished by acute (minutes) (9) and chronic (\(>3\) h Figure 3B) kinetics, respectively. They may represent sequential temporal arms of an integrated antiproliferative strategy in which acute cytostasis, silenced by phosphodiesterase-dependent desensitization of cGMP signaling (10), is propagated by CaR-dependent signaling to ensure enduring colon tumor cytostasis induced by GCC. Interestingly, CaR signaling also promotes phosphodiesterase-dependent hydrolysis of cGMP (39), a mechanism that may contribute to desensitize cGMP-mediated inhibition of DNA synthesis and represents a negative feedback loop for cGMP-induced CaR.

First discovered in parathyroid cells where it senses blood Ca\(^{2+}\) levels and regulates parathyroid hormone release (40), CaRs are expressed in many cell types, including osteoclasts, neurons and hematopoietic cells (23,41,42). In colonocytes, CaRs sense 1–10 mM Ca\(^{2+}\)o in fecal water by interacting with the N-terminal extracellular domain, inducing intracellular signaling through heterotrimeric G proteins (23,43). One emerging function of CaRs in colonocytes is the regulation of proliferation and differentiation and its putative role as a tumor suppressor (22,24,44). Although the CaR gene is not lost or mutated in colon cancer (33), CaR expression is inexplicably reduced during disease progression (25,26,33). The present finding that GCC regulates the posttranscriptional expression of CaRs in normal and malignant intestinal cells offers a mechanistic explanation for that observation since GCC signaling is silenced early during colorectal tumorigenesis following loss of ligand expression (11–13). Accordingly, elimination of GCC signaling in mice abolished the expression of CaRs in enterocytes (Figure 4). Moreover, CaRs are primarily expressed in the apex and central regions of human crypts (26) where expression of endogenous GCC ligands and cGMP levels are greatest (45,46), suggesting that CaR signaling may be conditionally regulated along the crypt–villus axis by GCC- and cGMP-dependent mechanisms. Both the common abilities of CaRs and GCC to promote the transition from proliferation to differentiation and inhibit intestinal carcinogenesis may represent convergent, rather than parallel, signaling pathways. Finally, although they remain to be fully characterized, GCC effects on CaRs, including induction of translation, posttranslational processing or trafficking to cell membranes, represent a previously unrecognized mechanism of transcriptionally independent regulation of CaR surface expression and signaling (47).

Beyond CaR, Ca\(^{2+}\)o opposes tumorigenesis by forming benign insoluble complexes with toxic ionized fatty acids and bile acids (48) and promoting cts-dimerization of E-cadherin molecules underlying cell–cell adhesion (49) and growth suppression (50). This latter mechanism, which occurs at Ca\(^{2+}\)o > 0.5 mM (49) and induces the function of p27 (50), may explain, in part, the cytostatic effects of high Ca\(^{2+}\)o in colon cancer cells not exposed to ST observed here. Indeed, these cells were not affected by CaR antisense delivery (Figure 5A) or application of CaR agonists (Figure 5C), indicating that in the absence of GCC activation CaRs are functionally silent in colon cancer cells. Loss of GCC (11–13) and CaR (25,26,33) signaling, in turn, may underlie the resistant phenotype of colon cancer cells to Ca\(^{2+}\)o-induced cytostasis (20,31). These observations are significant since levels of 3–10 mM Ca\(^{2+}\)o required for significant CaR activation (34), are typically achieved in the fecal colonic water as a result of dietary Ca\(^{2+}\) intake, absorption and secretion (19,22). Moreover, allosteric CaR activators present in the intestinal lumen such as l-amino acids (23) may further increase the CaR sensitivity for Ca\(^{2+}\)o. Importantly, CaRs sense other polyvalent cations (i.e. Gd\(^{3+}\), Mg\(^{2+}\), N\(^{2+}\) and polylysine), including polyamines (spermine, spermidine and putrescine) endogenously produced by colonic bacteria (23), suggesting that GCC-induced CaRs may subserve diverse tumor suppressor pathways in intestine.

Strategies for cancer control include chemoprevention and chemotheraphy. In contrast to its endogenous ligands, GCC is universally overexpressed in colorectal tumors (51,52), and oral administration of GCC ligands is a novel targeted approach to prevent tumor initiation and arrest disease progression in patients (3,4,9). Limitations to these therapies include adverse effects associated with the use of diarrheagenic bacterial enterotoxins (7,37,38). Conversely, dietary Ca\(^{2+}\) supplementation has been proposed as a chemoprevention strategy against colon cancer (53). However, caution has been suggested based on the reduced efficacy of Ca\(^{2+}\o\), to inhibit intestinal cell proliferation following neoplastic transformation probably reflecting reduced CaR expression (33). In that context, dietary Ca\(^{2+}\o\) could potentially promote growth of Ca\(^{2+}\o\)-insensitive tumor cells while suppressing proliferation of normal adjacent cells, which retain Ca\(^{2+}\o\) sensitivity (22). The present observations that GCC preconditioning restores antitumorigenic CaR signaling in human colon carcinoma cells may offer a solution to the limitations of Ca\(^{2+}\o\) supplementation strategies. Oral Ca\(^{2+}\o\) therapy, in turn, may prevent adverse effects of GCC-targeted strategies because activation of CaR opposes secretory diarrhea by bacterial enterotoxins (39). Taken together, these data suggest that combinatorial therapies including dietary Ca\(^{2+}\o\) supplementation and GCC ligand replacement represent a previously unrecognized paradigm for the prevention and treatment of human colorectal cancer.

**Supplementary material**

Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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