Sepiapterin Ameliorates Chemically Induced Murine Colitis and Azoxymethane-Induced Colon Cancer[§]

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ABSTRACT

The effects of modulating tetrahydrobiopterin (BH4) levels with a metabolic precursor, sepiapterin (SP), on dextran sodium sulfate (DSS)-induced colitis and azoxymethane (AOM)-induced colorectal cancer were studied. SP in the drinking water blocks DSS-induced colitis measured as decreased disease activity index (DAI), morphologic criteria, and recovery of Ca²⁺-induced contractility responses lost as a consequence of DSS treatment. SP reduces inflammatory responses measured as the decreased number of infiltrating inflammatory macrophages and neutrophils and decreased expression of proinflammatory cytokines interleukin 1 β (IL-1 β), IL-6, and IL-17A. High-performance liquid chromatography analyses of colonic BH4 and its oxidized derivative 7,8-dihydrobiopterin (BH2) are inconclusive although there was a trend for lower BH4:BH2 with DSS treatment that was reversed with SP. Reduction of colonic

cGMP levels by DSS was reversed with SP by a mechanism sensitive to 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a specific inhibitor of the NO-sensitive soluble guanylate cyclase (sGC). ODQ abrogates the protective effects of SP on colitis. This plus the finding that SP reduces DSS-enhanced protein Tyr nitration are consistent with DSS-induced uncoupling of NOS. The results agree with previous studies that demonstrated inactivation of sGC in DSS-treated animals as being important in recruitment of inflammatory cells and in altered cholinergic signaling and colon motility. SP also reduces the number of colon tumors in AOM/DSS-treated mice from 7 to 1 per unit colon length. Thus, pharmacologic modulation of BH4 with currently available drugs may provide a mechanism for alleviating some forms of colitis and potentially minimizing the potential for colorectal cancer in patients with colitis.

Introduction

The link between inflammation and cancer was proposed 150 years ago when Virchow argued that cancers arise at sites of chronic inflammation (Balkwill and Mantovani, 2001). The underlying mechanisms involve the recruitment of inflammatory cells and the generation of cytokines and enzymes that facilitate the development of the inflammatory microenvironment of the tumor and which are associated with other inflammatory diseases such as colitis. Important roles for reactive oxygen/nitrogen species (ROS/RNS) in tumor initiation and progression also have been clearly established (Wink et al., 1998; Westbrook and Schiestl, 2010).

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Expression of inducible NOS (NOS-2) in both epithelial and invading inflammatory cells is enhanced in colitis, contributing to nitro-oxidative stress throughout carcinogenesis and providing one explanation for the enhanced protein Tyr nitration observed in these colorectal cancer (CRC) tissues (Itzkowitz and Yio, 2004). Of potential significance to colitis is the nitration and inhibition of the Ca_v1.2 calcium channel of colonic smooth muscle and its role in motility changes resulting from colitis (Ross et al., 2007; Kang et al., 2010). However, equally elevated levels of protein nitration have been observed in chemically induced colitis of wild-type and NOS-2 knockout mice, and no difference in CRC incidence has been observed in comparing these mice (Seril et al., 2007). One explanation is a compensatory mechanism involving increased expression of other NOS isoforms such as endothelial NOS (NOS-3).

For all NOS isoforms, a transfer of electrons is coupled with the oxidation of arginine by a mechanism in which the cofactor tetrahydrobiopterin (BH4) donates electrons to the NOS ferrous dioxygen complex to initiate oxidation. The concentration ratio of BH4 to its oxidation product, 7,8-dihydrobiopterin

ABBREVIATIONS: ANOVA, analysis of variance; AOM, azoxymethane; BH2, 7,8-dihydrobiopterin; BH4, tetrahydrobiopterin; CRC, colorectal cancer; DAI, disease activity index; DAPI, 4',6-diamidino-2-phenylindole; DSS, dextran sodium sulfate; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; MPO, myeloperoxidase; NO, nitric oxide; NF- κ B, nuclear factor- κ B; NOS, nitric-oxide synthase; O₂⁻, superoxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PCR, polymerase chain reaction; PKG, protein kinase G; RNS, reactive nitrogen species; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SP, sepiapterin.

(BH2), is critical because BH2 binds with equal affinity for NOS but in a catalytically nonproductive manner. When the BH4:BH2 ratio is much greater than 1, NOS catalysis generates NO and citrulline, and NOS is said to be “coupled.” The primary target of NO is soluble guanylate cyclase (sGC), which stimulates cGMP synthesis and activates protein kinase G (PKG). This pathway is important in smooth muscle relaxation and synaptic transmission. In the vasculature, the NO-sGC-PKG pathway is anti-inflammatory. In colon carcinoma cell lines, increased PKG expression reduces cell proliferation and enhances apoptosis (Deguchi et al., 2005; Cen et al., 2008; Kwon et al., 2010).

When the BH4:BH2 ratio is low, as in chronic inflammatory conditions, coupling is less efficient and superoxide (O_2^-) is produced rather than NO. Under these conditions, NOS is uncoupled and has been called peroxynitrite synthase (Stuehr et al., 2001; Mikkelsen and Wardman, 2003; Alp and Channon, 2004; Cai et al., 2005). Peroxynitrite is a Tyr-nitrating free radical. NOS uncoupling has been extensively investigated in vascular diseases, and a synthetic BH4 (Kuvan) is currently in clinical trials for these diseases that has been approved for use in the treatment of some forms of phenylketonuria (Forstermann, 2010; Schmidt et al., 2010).

A previous study demonstrated that dietary supplementation with nitrite ameliorated DSS-induced colitis, possibly by bypassing NOS activity and using an alternative mechanism of NO generation involving the reduction of nitrite (Lundberg and Weitzberg, 2009; Ohtake et al., 2010). Our study examined the effect of modulating BH4 levels by dietary pretreatment of animals with sepiapterin (SP), a metabolic precursor of BH4, thereby enhancing the NO-sGC-PKG pathway and abrogating DSS induction of colitis and CRC.

Materials and Methods

Mouse Model of Colitis. Colitis was induced by the addition of 2.5% dextran sodium sulfate (DSS; USB Corporation, Cleveland, OH) to the drinking water of 5- to 6-week-old C57BL/6 male mice for a period of 7 days. The mice designated for SP treatment (Schircks Laboratories, Jona, Switzerland) received 4 mg/100 ml SP in their drinking water continually starting 3 days before DSS treatment. The drinking water was delivered with calibrated liquid feeding tubes from Bio-Serv (Frenchtown, NJ). None of the supplements affected the amount of water consumed by the animals, which was on average 4 ml per day. The dose of SP, approximately 0.64 mg/kg per day, was significantly less than that used in previous studies on vascular function, whether provided in food pellets (Pannirselvam et al., 2002) or in the drinking water (Shimazu et al., 2011).

This dosage was selected on the basis of preliminary experiments, demonstrating that it increased the BH4:BH2 levels in and was cytotoxic to different tumor cell lines grown as xenografts or in tissue culture (C. S. Rabender and R. B. Mikkelsen, unpublished data). All animals were monitored daily for weight, stool consistency, and the presence of blood in the excreta (Sasaki et al., 2005).

The disease activity index (DAI) was determined by combining the scores for weight loss, stool consistency, and bloody excreta as follows: weight loss score = 0: <1%, 1: 1–5%, 2: 5–10%, 3: 10–15%, 4: >15%; stool consistency score = 0: normal, 2: loose, 4: diarrhea; and blood in excreta score = 0: normal, 2: reddish, 4: bloody. Long-term colitis/carcinogenesis was induced by the intraperitoneal injection of azoxymethane (AOM) (10 mg/kg) 7 days before the onset of DSS treatments; 3-week courses of DSS were provided, as described earlier, separated by 2 weeks of DSS-free water. The animals were killed 3 weeks after the final DSS treatment, which was 77 days after AOM treatment.

The mice designated for SP treatment received 4 mg/100 ml SP in their drinking water continually starting 3 days before the DSS treatment. To inhibit sGC, animals were injected intraperitoneally daily with 20 mg/kg 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) as described by Tseng et al. (2011).

All animals were monitored daily for weight, stool consistency, and the presence of blood in the excreta. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and conformed to the guidelines established by the National Institutes of Health, protocol numbers AM10080 and AM10185.

BH4/BH2 Analysis. The purification and analysis of colonic biopterins were modifications of a method previously described elsewhere (Fukushima and Nixon, 1980; Sawabe et al., 2004). The frozen distal section of colons was homogenized in 10 volumes of 0.1 N HCl and was centrifuged at 13,200*g* for 20 minutes at 4°C. Aliquots of supernatant were mixed with 0.625 volumes of an acid-iodine solution (2% I_2 , 3% KI in 0.1 N HCl) or alkaline-iodine (2% I_2 , 3% KI in 0.2 N NaOH) for oxidation under acid or alkaline conditions, respectively (1 hour in the dark at room temperature). After we added 0.5 volumes of 2.5% ascorbic acid in 0.4 N perchloric acid, a clear solution was obtained by centrifugation for 10 minutes at 10,000*g*. BH4 and BH2 were measured by high-performance liquid chromatography using a fluorescence detector (excitation = 350 nm; emission = 450 nm). The solid phase was Partisphere RTF C18 4.6 × 250 mm (Whatman, Clifton, NJ), and the mobile phase was 7% methanol. BH4 and BH2 were quantified as described elsewhere (Sawabe et al., 2004).

cGMP and Myeloperoxidase Enzyme-Linked Immunosorbent Assay. For cGMP, frozen colons were homogenized in 10 volumes 0.1 N HCl and were centrifuged at 13,200*g* for 20 minutes at 4°C. The resulting supernatant was analyzed for cGMP using the cGMP Enzyme Immunoassay Kit (Cayman Chemical Co., Ann Arbor, MI) following the manufacturer's instructions. For analysis of myeloperoxidase (MPO), colons were homogenized in the nondenaturing cell lysis buffer from Cell Signaling Technology (Danvers, MA) and MPO levels measured with the Raybiotech Enzyme-Linked Immunosorbent Assay (ELISA) kit (Norcross GA) for mouse MPO.

Isometric Tension Recording. Approximately 1.5-cm strips of distal colon were suspended in the longitudinal direction in an organ bath containing 15 ml of Krebs solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH_2PO_4 , 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, and 2.5 mM $CaCl_2$), bubbled continuously with carbogen (95% O_2 and 5% CO_2) at 37°C under a resting tension of 1 g and equilibrated for a period of 1 hour. Isometric contractions were recorded by a force transducer (model GR-FT03; Radnoti, Monrovia, CA) connected to a personal computer using Acqknowledge 382 software program (BIOPAC Systems, Santa Barbara, CA).

After equilibration in the Krebs solution, tissues were incubated for 30 minutes in Ca^{2+} -free high-potassium solution (80 mM) in which equimolar NaCl was replaced by KCl containing 0.1 mM EGTA and changed every 15 minutes. Cumulative dose-dependent contraction responses to $CaCl_2$ (10 μ M to 10 mM) were performed in distal colon strips depolarized by calcium-free high- K^+ (80 mM) physiologic saline solution (without EGTA). The cumulative concentration-dependent increases in tissue tension by $CaCl_2$ were analyzed among tissue strips isolated from the control mice and those treated with DSS, DSS with SP, and SP alone.

Immunohistopathology. Colons were excised from animals, flushed with phosphate-buffered saline, cut longitudinally, rolled into “Swiss rolls,” and immediately flash-frozen in liquid N_2 . Frozen Swiss rolls were embedded in Tissue-Tek optimum cutting temperature (OCT) freezing medium (Sakura Finetek USA, Torrance, CA), and cryosections were prepared.

For macrophage detection, frozen sections were fixed in ice-cold acetone for 10 minutes. After donkey serum blocking for 60 minutes at room temperature, the sections were stained with primary mAb for F4/80 (5 μ g/ml; AbD Serotec, Raleigh, NC) overnight at 4°C, followed by incubation with Alexa Fluor 594-labeled donkey anti-rat secondary

IgG (2 μ g/ml; Invitrogen, Carlsbad, CA) at room temperature for 1 hour. A negative staining control was performed by incubation with isotype control Abs.

The coverslips were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were captured using the Ariol Digital Pathology Platform (Leica Microsystems, Buffalo Grove, IL). Protein nitroTyr staining of 6- μ m frozen, OCT-embedded tissues was analyzed by immunofluorescence microscopy with mouse biotin labeled anti-nitroTyr (Cayman Chemical Co.) using a biotin-avidin kit for mouse primary antibodies from Vector Laboratories.

For H&E staining, cryosections (6 μ m) were fixed in 4% para-formaldehyde, submerged in modified Mayer's hematoxylin, destained in acid ethanol, stained with eosin, dehydrated, and mounted with Permount. Images were captured and analyzed using the Ariol Digital Pathology Platform.

Gene Expression Analysis. Colons were excised, flushed with phosphate-buffered saline, and flash-frozen using liquid N₂. The Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) was used per the manufacturer's instructions to extract mRNA. We generated cDNA using the Invitrogen SuperScript III First-Strand Synthesis System for reverse-transcription polymerase chain reaction (PCR) per the manufacturer's instructions using 1 μ g of total RNA per reaction and Oligo(dT)₂₀ primers. For quantitative PCR analysis, transcription profiles of *il17a*, *il1b*, and *il6* were assessed on an ABI prism 7900HT Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The primers and FAM-labeled probe sets were obtained as predeveloped assay reagents from Applied Biosystems: *il17a*, Mm00439618_m1, *il1b*, Mm01336189_m1, *il6*, and Mm99999064_m1. The PCR was started with 2 minutes at 50°C, and then an initial 10 minutes' denaturation at 94°C, followed by a total of 40 cycles of 15-second denaturation at 94°C, and 1 minute of annealing and elongation at 60°C.

All measurements were performed in triplicate wells and were repeated three times. Gene expression was quantified relative to the expression of the housekeeping gene β -actin, and was normalized to that measured in control cells by standard $2^{(-\Delta\Delta CT)}$ calculation.

Statistical Analysis. For contraction measurements, the data from different groups were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's post test. $P \leq 0.05$ was considered statistically significant. For all other experiments, data are shown as mean $\pm$ S.E.M. with P values calculated by Student's t test or in the

Fig. 1. Disease activity in mice with experimental colitis. Disease activity in animals receiving 2.5% DSS in their drinking water was statistically significantly reduced in animals that also received 4 mg/100 ml SP in their drinking water. Attenuated disease was observed as measured both by animal weight (A) and disease activity index (B). Data are presented as mean $\pm$ S.E.M., $n = 5$ per group.

case of the microscopic analysis by a one-way ANOVA with each treatment group as the only effect. The difference of least square mean for each group was tested using a F test for statistical significance.

Fig. 2. H&E staining of colons from control and treated mice. H&E-stained frozen sections of colon Swiss rolls. Swiss roll preparations allow the visualization of the whole colon distal (D) to proximal (P) in a single section.

Results

SP Treatment Ameliorates DSS-Induced Colitis. To determine the effect of SP upon experimental colitis, the animals were placed in four groups of drinking water doped with DSS, SP, DSS + SP, or untreated water. They then were monitored for the development of colitis. Colitis was measured by daily recordings of weight, stool consistency, and the presence of blood in the excreta. As shown in Fig. 1A, by day 7, the animals receiving DSS had lost 14% of their starting body weight. The weight loss in the DSS-treated animals was prevented by the addition of SP to the drinking water. Likewise, the DAI (Fig. 1B) was statistically significantly increased in only the DSS-treated animals at day 7.

The development of DSS-induced colitis and its partial mitigation by cotreatment with SP was also monitored by H&E staining of frozen sections of colons from treated animals. As shown in Fig. 2, a loss of villi and crypts was observed in the colon of mice treated with DSS alone; cotreatment with SP mostly mitigated this change in colonic morphology. Separation of the muscle and *muscularis mucosae* was predominantly observed in both the DSS-treated colons but also was observed to some degree in SP colons, indicating some mild colitis in

DSS + SP colons that was not detected by assessment of the disease activity index.

SP Rescues the Reduced Calcium-Induced Contraction in Murine Colitis. Cumulative addition of CaCl_2 induced a concentration-dependent contraction in both control and inflamed (DSS-treated) distal colon depolarized by Ca^{2+} -free high potassium (80 mM) physiologic saline solution. DSS treatment statistically significantly ($P \leq 0.01$) reduced the overall calcium-induced contraction curve (Fig. 3, A–C), as analyzed by two-way ANOVA [$F(1,28) = 8.30$]. In vivo treatment of SP reversed the reduced CaCl_2 -contraction in the DSS-treated group (Fig. 3, B and E) without affecting the contractility in the control group (Fig. 3D) [DSS + SP, $F(1,28) = 26.65$; SP, $F(1,28) = 0.42$; two-way ANOVA; $n = 3$ per group]. The efficacy of CaCl_2 was statistically significantly ($P \leq 0.001$) enhanced by SP treatment in the DSS-treated group, unlike in the otherwise untreated animals.

The DSS-Induced Inflammatory Response Is Reduced by SP Treatment. DSS treatment is known to elicit an inflammatory response that includes the recruitment of infiltrating immune cells such as macrophages. As SP treatment is expected to be anti-inflammatory, we stained

Fig. 3. Colon contractility in DSS-induced colitis. Representative isometric tension recording of CaCl_2 -induced concentration-dependent contraction of distal colon tissue strips from (A) control mice (B) mice treated with DSS and DSS + SP. The right panel displays a graphic representation and comparison of concentration-dependent CaCl_2 -induced increase in tension of colon muscle strips: (C) control versus DSS, (D) control versus SP, and (E) DSS versus DSS + SP. Note that inflammation decreased the contraction while treatment with SP increased the CaCl_2 -induced contraction in inflamed tissues. Data are expressed as mean $\pm$ S.E.M., and were analyzed by two-way ANOVA followed by Bonferroni post test, $n = 3$ per group.

colon sections for the presence of macrophages. The top row of images of Fig. 4 [F4/80 + DAPI (4',6-diamidino-2-phenylindole)] show that infiltration of inflammatory macrophages was observed throughout the length of the mouse colon after DSS treatment and this is mostly reversed by inclusion of SP in the drinking water of mice. Higher magnifications are also shown in the lower rows of images. Statistical significance was established by semiquantitative analysis of the images of three images from four mice in each treatment group, as shown in Supplemental Fig. 1A. In addition, the measurements of MPO protein by ELISA demonstrated elevated levels of neutrophils after DSS treatment, which were reversible with coadministration of SP (Supplemental Fig. 1B). These results confirm that DSS treatment elicits an inflammatory response and that it is at least partially reversible with SP.

To determine how SP treatment reduced macrophage recruitment in DSS-treated colons, we performed a gene expression analysis to determine the expression of proinflammatory and anti-inflammatory cytokines. As shown in Fig. 5, DSS treatment elicited significant increases in the expression of mRNA for the proinflammatory cytokines IL-1 β , IL-6, and IL-17A. This increased cytokine expression was blocked by the addition of SP to the drinking water. We also assayed for the anti-inflammatory cytokine IL-37 because this cytokine has been shown to protect the mouse colon from DSS-induced colitis (McNamee et al., 2011). However, we did not detect IL-37 expression in any of the colon samples tested. The observed changes in cytokine expression indicate that SP acts by disrupting both the acute phase response and delayed-type immune reaction.

SP Blocks the DSS-Induced Reduction in Colon cGMP Levels. To determine whether DSS-induced inflammation decreases NOS coupling and thus NO production, we assayed the BH4:BH2 ratio after 7 days of DSS treatment. Colonic cGMP

levels were also measured at this time as an indirect measure of coupled NOS activity. Figure 6 shows the results from one experiment where the BH4:BH2 was measured on day 7 of the DSS treatment. The trend of lower BH4:BH2 with DSS treatment was observed, but the magnitude of the change never reached statistical significance due to the high variability between the samples. SP treatment with or without DSS, however, increased the BH4:BH2 ratio.

We did not observe any significant change in the BH4/BH2 ratio with DSS treatment, but the production of cGMP—an indirect measure of NO production—was statistically significantly reduced in the DSS-treated colons ($P < 0.01$) (Fig. 7B). The DSS-induced reduction in the levels of cGMP was reversed by SP treatment. SP alone statistically significantly increased the cGMP production relative to the control animals.

Fig. 4. SP treatment decreases macrophage and neutrophil colonic infiltration into DSS-treated animals. Cryosections of colon were stained with rat anti-mouse mAbs for F4/80, followed by incubation with Alexa 594-conjugated donkey anti-rat secondary IgG. Scale bar, 100 μ m. The entire colon is shown as Swiss rolls in the top row. Typical 20X magnification images are shown in the bottom rows. The bottom row shows the control images without the primary antibody.

Fig. 5. Increased proinflammatory cytokine expression of diseased colons is reduced by SP treatment. Effect of SP cotreatment on *il1b*, *il6*, and *il17a* gene expression levels in the diseased colons was assayed by quantitative real-time reverse-transcription PCR and normalized to the β -actin gene in the control group (C). Each bar represents the cDNA from one animal analyzed in triplicate, with the error bars representing the mean $\pm$ S.E.M. for the triplicate samples. &: not detectable.

Fig. 6. BH4:BH2 levels in colons from control and treated mice. BH4 and BH2 were measured by high-performance liquid chromatography analysis, as described under *Materials and Methods*. Each bar represents the mean $\pm$ S.E.M. of four colons for each treatment.

cGMP is also generated by a NO-independent but Ca^{2+} -requiring membrane-bound guanylate cyclase. That SP treatment reverses the effect of DSS on colon cGMP levels suggests that increased NO generation is required. To further

Fig. 7. The sGC inhibitor ODQ inhibits the SP mitigation of DSS-colitis and blocks SP-induced cGMP increases in DSS-treated colons. (A) DAI was measured as described in Fig. 1. (B) cGMP assayed by ELISA shows decreased cGMP production in DSS-treated whole colons that is restored when animals also received SP. cGMP is an indirect measure indicating decreased NO production in DSS-treated colons. Data are presented as the mean $\pm$ S.E.M., four colons per treatment group, with * $P < 0.05$ for DSS and SP compared with control or DSS + SP.

test this mechanism, we used the sGC inhibitor ODQ, which is an irreversible inhibitor of sGC. In these experiments, ODQ was delivered i.p. on a daily basis with changes in the SP-containing water. As shown in Fig. 7B, ODQ blocked the activity of SP, preventing the decrease in cellular cGMP in the colons of mice treated with DSS. Parallel experiments were performed in which the DAI of the animals were measured. As shown in Fig. 7A, ODQ also prevented the SP-induced recovery in the DAI of the DSS-treated animals.

SP Inhibits DSS-Induced Protein Tyr Nitration. Previous investigators have called uncoupled NOS a peroxynitrite synthase. Thus, if uncoupling of NOS occurs with DSS treatment, one would expect increased protein Tyr nitration, as has been observed in numerous studies with DSS-induced colitis (Yasui et al., 2007; Westbrook and Schiestl, 2010). As shown in Fig. 8, DSS treatment enhanced colonic protein Tyr nitration as detected by immunofluorescence, and this was abrogated by coadministration of SP. Statistical analysis demonstrated a >5 -fold increase in nitroTyr staining relative to DAPI fluorescence in the colons from DSS-treated animals compared with controls and mice treated with SP + DSS ($P < 0.01$, $n = 3$ animals per treatment group, three images per animal). As shown in image N, which is a magnification of image J, the increased nitroTyr staining was due not only to infiltrating inflammatory cells but also to colonic epithelial cells. This result along with the observed changes in cGMP and their reversibility by treatment with the BH4 precursor SP are consistent with the uncoupling of NOS in DSS-induced colitis.

SP Partially Protects against AOM/DSS-Induced Adenocarcinoma. Given that SP protects against the development of DSS-induced colitis, we tested the hypothesis that this protection may extend to preventing the development of AOM/DSS-induced CRC. Animals received a dose of AOM followed by three 1-week courses of DSS with or without SP cotreatment. After 70-days of treatment, colons were excised and assayed morphologically for tumor development. Individual single treatments with AOM or DSS did not induce cancer formation, as previously described by Neufert et al. (2007). As seen in Fig. 9A, the combined AOM + DSS treatment induced tumor formation that was statistically significantly reduced by cotreatment with SP ($P < 0.05$). In the 10 animals treated with AOM + DSS as described, the number of tumors per distal colon ranged between 2 and 8. In the 10 animals in the AOM + DSS + SP group, the number of tumors per colon ranged between 0 and 3. The modest increase (15%) in colon length with SP treatment was insufficient to account for the difference observed between the two groups. Interestingly, the SP cotreatment did not decrease the average size of those tumors that did develop (Fig. 9B), which suggests that SP acts to limit tumor initiation rather than to restrict tumor progression. However, because the tumors were harvested at a relatively late time after initiation, the effects of SP on tumor progression would have been missed.

Discussion

By multiple criteria, we have demonstrated that cotreatment of animals with SP counteracts the colitis-inducing effects of DSS. Thus, SP treatment blocks the increased DAI and the loss of microvilli and crypts, and recovers the Ca^{2+} -induced

Fig. 8. NitroTyr staining is increased in diseased colons by a mechanism inhibited by SP. Images (A–F) DAPI + fluorescein-Avidin-stained Swiss rolls. (G–L) Corresponding fluorescein-Avidin stained sections with the blue DAPI fluorescence off. Lanes A + G (no primary anti-nitroTyr) and B + H (+ primary anti-nitroTyr) are adjacent cryostat sections from the colons of untreated animals; C + I (no primary anti-nitroTyr) and D + J (+ primary anti-nitroTyr) are adjacent colon sections from DSS-treated animals; E + K (no primary anti-nitroTyr) and F + L (+ primary anti-nitroTyr) are adjacent sections from the colons of DSS animals rescued with SP. (M and N) Magnifications of sections from the adjacent images I and J showing infiltration of inflammatory cells into the colon of DSS-treated animals as well as increased nitroTyr staining of epithelial cells.

contractility responses lost as a consequence of DSS treatment. In addition, our study demonstrates that SP treatment reduces the inflammatory response measured as either an increased number of infiltrating inflammatory macrophages and neutrophils or increased expression of proinflammatory cytokines IL-1 β , IL-6, and IL-17A. Our studies also show that by minimizing inflammation, SP also decreased the numbers of tumors formed as a consequence of tumor initiation with the carcinogen AOM.

The initial hypothesis for this study was that DSS treatment would further promote the chronic inflammatory environment characteristic of colitis by reducing the BH4: BH2 ratio and uncoupling NOS activity, resulting in the generation of ROS/RNS. However, the changes we observed in the colonic BH4: BH2 ratio, whereas consistent with the

hypothesis were not statistically significant. Even though a significant decrease in the BH4: BH2 ratio was not measured in the colons from DSS-treated animals, the observed changes in cGMP levels and protein Tyr nitration after DSS treatment and their reversibility with SP are consistent with NOS uncoupling.

A previous study demonstrated that DSS-induced colitis is associated with inhibition of colonic sGC activity, leading to lower cGMP levels (Van Crombruggen et al., 2008; Schmidt et al., 2012). The underlying mechanism for the inhibition was not established, but it did not involve changes in either nNOS mRNA or protein levels. Those investigators argued that the reduced sGC activity was associated with a decreased colonic responsiveness to nitrergic stimuli. More recently, a mechanism for the inactivation of sGC in inflammatory conditions

Fig. 9. Reduced AOM/DSS-induced colon tumorigenesis in SP-treated animals. (A) Number of tumors identified per unit length of colon in Swiss rolls. The results represent the mean + S.E.M. for $n = 10$ for each treatment group. * $P < 0.05$. (B) Relative size of tumors evaluated digitally from H&E-stained sections.

has been shown to involve oxidation of the heme cofactor of sGC (Schmidt et al., 2012). Protection of sGC from oxidative inactivation in vitro was achieved by culturing cells with BH4 or its precursors BH2 and SP. This latter mechanism can occur without changes in the BH4:BH2 ratio or NOS coupling.

Measurements of total BH4:BH2 ratio in the whole distal colon do not necessarily reflect changes in the different cellular compartments of the colon that may have an impact on colitis. For example, DSS-induced NOS uncoupling in nitrergic neurons by disrupting the normal nicotinic-nitrergic communication could be by inhibiting cholinergic signaling increase secretion relative to absorption and thereby stimulate diarrhea (Green et al., 2004). The immunofluorescent images of protein Tyr nitration also suggest localized changes in NOS activity with DSS treatment.

Colitis is a consequence of the relative changes in secretory and absorptive functions of the colon that result in changes in gastrointestinal motility. Genetic, environmental, microbial, and immunologic factors all play a role, ultimately resulting in loss of barrier function and the pathologic invasion of inflammatory cells into the mucosa and increased expression of inflammatory cytokines. It remains unclear how these different factors relate to one another in initiation and progression of colitis and where NO signaling and biopterins such as BH4 and SP contribute to the development and prevention of colitis. One mechanism that deserves consideration also involves sGC and its role in inhibiting the recruitment of inflammatory cells to sites of tissue injury (Ahluwalia et al., 2004). In endothelial cells, sGC activity inhibits inflammatory cell recruitment by blocking P-selection expression (Ahluwalia et al., 2004). Thus, besides restoring maintaining barrier function and the normal balance of secretion and absorption, by protecting sGC from oxidative inactivation SP may also mitigate the effects of DSS-induced colitis by preventing the recruitment of inflammatory cells.

The three cytokines we examined—IL-1 β , IL-6, and IL-17—have all been associated with DSS-induced colitis and with colon CRC (Kaler et al., 2009; Hyun et al., 2010; Waldner et al., 2012). One unifying feature for all three cytokines is their transcriptional regulation by nuclear factor κ B (NF- κ B) (Funakoshi et al., 2012). Dehydroxymethylepoxyquinomicin, a relatively specific inhibitor of NF- κ B, inhibits the mRNA expression of IL-1 β , IL-6, tumor necrosis factor α (TNF- α), IL-12p40, IL-17A, and monocyte chemoattractant protein 1 (MCP-1) and also suppresses both DSS and trinitrobenzene sulfonic acid (TNBS)-induced colitis.

With respect to how SP inhibits proinflammatory cytokine expression, one mechanism is based on a previous study showing that low inflammatory levels of RNS result in the nitration Tyr181 of I κ B α , the inhibitor protein of NF- κ B, dissociating the complex and facilitating translocation of active transcription factor p65/p50 into the nucleus (Yakovlev et al., 2007). SP treatment by increasing the BH4:BH2 ratio recouples NOS activity, enhancing NO generation and reducing the RNS generation necessary for Tyr nitration. Furthermore, NO-dependent S-nitrosylation of p65 also inhibits NF- κ B transcriptional activity (Marshall et al., 2004). Thus, recoupling of NOS potentially inhibits NF- κ B activity, leading to suppression of the inflammatory response critical for colitis and colitis-associated carcinogenesis (Sawabe et al., 2004; Atreya et al., 2008; Shaked et al., 2012).

Regardless of the mechanism of SP activity, these results suggest a potential approach in mitigating colitis and associated CRC by modulating colonic BH4/cGMP metabolism. One potential candidate is a synthetic BH4 (Kuvan), which is currently used for treatment of some types of phenylketonuria. Kuvan is also being examined in phase 2 trials for treatment of different inflammatory and vascular diseases (Burton et al., 2011; Cunningham et al., 2012).

Authorship Contributions

Participated in research design: Cardnell, Rabender, Akbarali, Wang, Mikkelsen.

Conducted experiments: Cardnell, Rabender, Alam, Howlett, Ross, Guo.

Performed data analysis: Cardnell, Rabender, Wang, Ross, Guo, Mikkelsen.

Wrote or contributed to the writing of the manuscript: Cardnell, Rabender, Akbarali, Wang, Mikkelsen.

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