Stimulation of colonic mucosal growth associated with oxidized redox status in rats

Junqiang Tian, Naohiro Washizawa, Li H. Gu, Marc S. Levin, Lihua Wang, Deborah C. Rubin, Simon Mwangi, Shanthi Srinivasan, Yuhao Gao, Dean P. Jones, and Thomas R. Ziegler

1Nutrition and Health Science Program, Graduate School of Arts and Science, Emory University, Atlanta; 2Department of Medicine and 3Center for Clinical and Molecular Nutrition, Emory University School of Medicine, Atlanta, Georgia; 4Department of Surgery, Toho University School of Medicine, Tokyo, Japan; and 5Department of Medicine, Washington University School of Medicine, St. Louis, Missouri

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Am J Physiol Regul Integr Comp Physiol 292: R1081–R1091, 2007. First published November 9, 2006; doi:10.1152/ajpregu.00050.2006—Limited data in animal models suggest that colonic mucosa undergoes adaptive growth following massive small bowel resection (SBR). In vitro data suggest that intestinal cell growth is regulated by reactive oxygen species and redox couples [e.g., glutathione (GSH)/glutathione disulfide (GSSG) and cysteine (Cys)/cystine (CySS) redox]. We investigated the effects of SBR and alterations in redox on colonic growth indexes in rats after either small bowel transection (TX) or 80% midjejunoeileal resection (RX). Rats were pair fed ± blockade of endogenous GSH synthesis with buthionine sulfoximine (BSO). Indexes of colonic growth, proliferation, and apoptosis in GSH/GSSG and Cys/CySS redox potentials (Eh) were determined. RX significantly increased colonic crypt depth, number of cells per crypt, and epithelial cell proliferation [crypt cell bromodeoxyuridine (BrdU) incorporation]. Administration of BSO markedly decreased colonic mucosal GSH, GSSG, and Cys concentrations in both TX and RX groups, with a resultant oxidation of GSH/GSSG and Cys/CySS Eh. BSO did not alter colonic crypt cell apoptosis but significantly increased all colonic mucosal growth indexes (crypt depth, cells/crypt, and BrdU incorporation) in both TX and RX groups in a time- and dose-dependent manner. BSO significantly decreased plasma GSH and GSSG, oxidized GSH/GSSG Eh, and increased plasma Cys and CySS concentrations. Collectively, these data provide in vivo evidence indicating that oxidized colonic mucosal redox status stimulates colonic mucosal growth in rats. The data also suggest that GSH is required to maintain normal colonic and plasma Cys/CySS homeostasis in these animal models.

Factors that influence colonic adaptive growth after SBR have been little studied (55). However, such information is important, given the critical role of the colon in determining nutritional status and clinical outcomes in patients with SBS (58). Recent research suggests that redox signaling is an important mode of signal transduction for growth and turnover of intestinal cells and other cell types (5, 26). Proteins that regulate cell proliferation, differentiation, and apoptosis may be regulated by reversible modification of the sulphydryl group on active cysteine residues (39), a process executed by a number of systems including reactive oxygen species (ROS) (15) and major redox couples with thiol/disulfide exchange functions [glutathione (GSH)/glutathione disulfide (GSSG), cysteine (Cys)/cystine (CySS), and reduced/oxidized thioredoxin (TRX)]. As an important antioxidant, GSH reacts directly with ROS and is a cofactor of glutathione peroxidase, which participates in the quenching of ROS (26). As an l-glutamyl-l-cysteinyl-glycine tripeptide, GSH is also important for cellular Cys delivery (23, 31) and serves as a Cys reservoir in plasma and tissues (26, 45).

Several in vitro and in vivo studies suggest that a more reducing redox status is associated with intestinal cell growth, while oxidation of cellular redox pools may inhibit growth responses and/or increase cellular apoptosis (5). Also, marked depletion of cellular GSH by buthionine sulfoximine (BSO), a potent and specific inhibitor of GSH synthesis, resulted in severe degeneration of colonic epithelial cells in intact mice which was prevented by enteral or parenteral GSH repletion (37). We designed the current study to examine the effect of GSH depletion on colonic and plasma GSH/GSSG and Cys/CySS redox pools and adaptive colonic growth in response to massive SBR in an established rat model of SBS. Our data show that BSO-induced depletion of GSH results in concomitant depletion of Cys and oxidation of GSH/GSSG and Cys/CySS redox potentials in colonic mucosa and plasma. Our data also indicate that oxidized colonic mucosal redox status is a stimulus for colonic mucosal growth in both intact and SBR rats.

MATERIALS AND METHODS

Animals and materials. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 191–260 g, were housed in individual cages in the animal care facility under controlled conditions.

Address for reprint requests and other correspondence: T. R. Ziegler, Suite GG-23, General Clinical Research Center, Emory Univ. Hospital, 1364 Clifton Rd., Atlanta, GA 30322 (e-mail: tzieg01@emory.edu).

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of temperature and humidity with a 12:12-h light-dark cycle. Animals were given free access to water and standard pelleted rat food (Laboratory Rodent Chow 5001; PMI Feeds, St. Louis, MO) during a 7-day acclimation period. The study protocol was approved by the Institutional Animal Use Committee of Emory University (Atlanta, GA). BSO [N-(butylthio)semi-S(S,S)-sulfoximine] and bromodeoxyuridine (BrdU) were purchased from Sigma-Aldrich (St. Louis, MO). The primary (mouse anti-BrdU) and secondary (biotinylated anti-mouse IgG, rat adsorbed) antibodies in BrdU incorporation assay were purchased from Dako (Carpenteria, CA) and Vector Laboratories (Burlingame, CA), respectively.

Operative procedures and experimental design. We explored effects of BSO, a specific inhibitor of glutamate-cysteine ligase (GCL), the rate-limiting enzyme for GSH synthesis, on colonic redox and growth indexes in our rat models. Animals were fasted overnight, before operation. The following morning (day 1), rats underwent laparotomy and either 80% jejunoileal SBR (RX) or small bowel transection (TX; sham operation), as previously described (55). In the RX animals, 80% of the jejunum and ileum was removed using defined landmarks (from 10 cm distal to the ligament of Treitz to 10 cm proximal to the ileocecal junction). In TX animals, the small intestine was transected at a point 10 cm proximal to the ileocecal junction and anastomosed. Rats were allowed access to water after operation and standard pelleted rodent food from day 2. Rats in other groups were pair fed with the amount of food consumed by RX rats with BSO treatment. Body weight and food intake were determined daily until death.

Three independent experiments were performed in this study. **Experiment 1** included four groups of rats, designated as TX/saline (Sal) for sham-operated rats without BSO treatment (n = 7), TX/BSO for sham-operated rats with BSO treatment (n = 7), RX/Sal for 80% SBR rats without BSO treatment (n = 6), and RX/BSO for 80% SBR rats with BSO treatment (n = 7). The animals underwent TX or RX on day 1. BSO treatment began on day 5, with BSO (ip) given at a dose of 2.5 mmol/kg twice daily and BSO (10 mM) in drinking water. Although RX rats may have less absorptive capability of luminal BSO than TX rats, overall magnitudes of decrease in mucosal and plasma GSH were similar in TX and RX rats. An equivalent volume of saline was injected (ip) twice daily in non-BSO-treated rats. **Experiment 2** was conducted to define potential dose responses to BSO and subsequent GSH depletion-induced colonic mucosal growth. After a series of dose-response experiments in intact rats (not shown), we studied TX or RX rats, treated as in experiment 1, but including an RX group receiving BSO at a dose of 2 mmol/kg, with no BSO in the drinking water (RX/BSOlow). This separate experiment thus included four groups: TX/SaI (n = 6), RX/Sal (n = 5), RX/BSO (n = 5), and RX/BSOlow (n = 5). Otherwise, the experimental procedures were identical to those outlined in experiment 1. **Experiment 3** was performed to determine whether BSO given at an earlier time period after bowel resection or transection would influence colonic mucosal growth. Rats were randomized by weight into three groups: TX/Sal, RX/Sal, and RX/BSO (n = 2–5 in each group). These rats underwent the same treatments as in experiment 1, except that BSO or saline was given just before the operation and continued as outlined above until death at either 2 or 4 days postoperation.

**Tissue collection.** On the morning of day 8 (7 days after operation), the rats were killed between 9 AM and 12 PM after an overnight fast. The colon was stripped of mesenteric and vascular connections and removed from the peritoneum. The colonic lumen was flushed with ice-cold saline to clear intestinal contents and suspended from a ring stand with a constant distal weight. The defined segments used for the endpoints of this study were collected sequentially at an equivalent site of each animal. The segments used for mucosal thiols analysis were longitudinally cut, and mucosa was obtained by gentle scraping with a glass slide. The mucosa was immediately placed in liquid nitrogen for further processing (51). Blood was drawn and processed for thiol determination as described (26).

**Histology.** Defined segments of colon were fixed with 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were examined for morphological damage by visual inspection. Colonic crypt depth (CD) was measured as an index of colonic mucosal growth. The number of cells per colonic crypt and per unit length (μm) of CD on longitudinal sections was determined as morphological growth indexes, as previously described (51). All parameters were measured in 15–20 representative and longitudinally oriented full-length crypts.

**Cyst cell proliferation.** Cyst cell proliferation was assessed using 5-BrdU incorporation to identify cells in the S phase of the cell cycle (51). Rats were injected (ip) with 120 mg/kg 5-BrdU (40 g/l 5-BrdU and 4 g/l 5-fluorodeoxyuridine) 90 min before death. 5-BrdU was detected with a monoclonal antibody and a streptavidin-biotin staining system. The number of labeled cells in at least 10 well-oriented longitudinal crypts of each sample was determined with a light microscope by an examiner blinded to study groups and reported as number of 5-BrdU-labeled cells per 1,000 total crypt cells counted.

**Apoptosis.** Apoptotic cells were determined in colonic crypts by an examination blinded to the study groups, using classic morphological changes, including nuclear condensation, perinuclear clearing, and cell shrinkage, as described (51). This method of identifying apoptotic cells, presently considered the reference standard by Potten (48), is highly precise if representative morphological changes are observed. We also performed fluorescent double staining using caspase-3 and terminal deoxuryridine nick-end labeling (TUNEL) as a secondary method to detect apoptosis. After deparaffinization and hydration, paraffin sections of colon were retrieved for antigens in a pressure cooker with sodium citrate (pH 6.0, 10 mM) for 10 min. After cooling, the sections were quenched in 3% H2O2 in methanol and then blocked with normal donkey serum. Caspase-3 was detected with rabbit anti-cleaved-caspase-3 IgG overnight at 4°C followed by a labeled streptavidin-biotin (LSAB) staining method consisting of successive application of secondary antibody-streptavidin, biotin-horseradish peroxidase, and cyanine-3-tyramide. We performed TUNEL immunohistochemistry using the in situ cell death detection kit, fluorescein, as described by the manufacturer (Roche Applied Science, Indianapolis, IN). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) to count total crypt cell number. The apoptotic index for both morphologically defined apoptotic cells and fluorescent staining with caspase-3 was calculated as the number of apoptotic cells per 1,000 crypt cells counted.

**Electron microscopy.** Defined colonic samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, washed with distilled water, and postfixed with 1% osmium tetroxide. The samples were then processed as described (26) and examined using a JEOL JEM-1210 LaB6 Transmission Electron Microscope at 80 kV.

**Thiol determination.** GSH, GSSG, Cys, CySS, and mixed disulfide of GSH and Cys were separated and quantified by integration of their HPLC peaks relative to the internal standard (γ-glutamyl glutamate) (26). Data are expressed for mucosal samples (nmol/mg protein) and for plasma samples (μM). The redox potential (Eh) for the GSH/GSSG and Cys/CySS couples was calculated from the respective concentrations using the Nernst equation (26).

**IGF-I expression by real-time RT-PCR.** Total RNA was isolated from defined colonic sections of rats from Experiment 2 by standard methods, and real-time RT-PCR was performed to determine expression of IGF-I mRNA. The primers used for IGF-I expression were 5′-CCG GAC CAG AGA CCC TTT G-3′ (forward) and 5′-GCC GTG TTC TGAGTA AAG TAA AA-3′ (reverse) (40). IGF-I mRNA was normalized against glyceraldehyde dehydrogenase (GAPDH) mRNA levels using the primers 5′-AAT GTA TCC GTG AGT CTT GGT-3′ (forward) and 5′-GCC TGC TCC ACC ACC TTC T-3′ (reverse) (53). Amplification reactions were performed in 50 μl of volume using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). IGF-I mRNA in colon was quantitated by real-time PCR using a GeneAmp 7000 system (PE Biosystems) as previously described (11). Disso-
Association curves revealed a single product in all cases. Fold change in cDNA concentration was calculated using the $2^{-\Delta\Delta C_T}$ method (where $C_T$ is threshold cycle) (11, 16, 32) with normalization of input using GAPDH.

**Statistical analysis.** The study was set up as a $2 \times 2$ factorial design, with operation (RX vs. TX) and BSO treatment (BSO vs. saline) as the two main factors. Two-way ANOVA was performed to determine the significance of the main effects and their interaction (significance defined as $P < 0.05$). One-way ANOVA was also used to detect significant intergroup differences ($P < 0.05$). In this case, the four study groups were compared post hoc using Fishers protected least significant difference test. In correlative studies, the strength of association was represented by Pearson correlation coefficient ($r^2$) and tested by ANOVA (significance defined as $P < 0.05$). The trend line and regression equation were determined using simple linear regression analysis. All statistics were conducted using SPSS software (Chicago, IL). Data are presented as means $\pm$ SE.

**RESULTS**

**Food intake and body weight.** In experiment 1, mean daily food intake during the study period was similar between groups [TX/Sal 10.6 $\pm$ 1.2, RX/Sal 11.6 $\pm$ 0.5, TX/BSO 11.8 $\pm$ 0.6, RX/BSO 12.6 $\pm$ 1.5 g/day; not significant (NS)]. The body weight of RX rats decreased following SBR but was similar to that of TX rats after study day 3. Mean daily body weight was similar between groups over time (TX/Sal 214.8 $\pm$ 2.0, RX/Sal 212.6 $\pm$ 2.8, TX/BSO 219.9 $\pm$ 3.8, RX/BSO 212.6 $\pm$ 4.1 g; NS). BSO-treated rats and their saline-treated controls exhibited similar daily food intake and changes in body weight from baseline to day 7, suggesting that BSO treatment did not alter whole body nutrient utilization. Similar values of food intake and body weight were observed in experiments 2 and 3 (data not shown).

**BSO treatment oxidizes GSH/GSSG $E_h$ in colonic mucosa and plasma.** BSO treatment during postoperative days 5–7 (experiment 1) in both TX and RX groups markedly decreased colonic mucosal GSH and GSSG concentrations, respectively (Fig. 1, A and B). This response was associated with a substantial 50- to 60-mV oxidation of GSH/GSSG $E_h$ (Fig. 1C). Two-factor ANOVA revealed a main effect for BSO to significantly decrease colonic and plasma GSH and GSSG concentrations and oxidize GSH/GSSG $E_h$ in colon and plasma in TX

![Figure 1](http://ajpregu.physiology.org/)

**Fig. 1.** Buthionine sulfoximine (BSO) treatment decreases colonic mucosal glutathione (GSH) and glutathione disulfide (GSSG) concentrations and oxidizes colonic GSH/GSSG redox potential ($E_h$). See experiment 1 in MATERIALS AND METHODS for details of operations and treatment regimens. GSH and GSSG concentrations were measured by HPLC, and GSH/GSSG $E_h$ was calculated using the Nernst equation. A: colonic mucosal GSH concentration. B: colonic mucosal GSSG concentration. C: GSH/GSSG $E_h$. D: correlation of colonic mucosal GSH concentration with GSH concentration in plasma. TX, transection; RX, resection; Sal, saline. #TX/Sal vs. TX/BSO, $P < 0.05$. †RX/Sal vs. RX/BSO, $P < 0.05$ by 1-factor ANOVA. *Main effect of BSO treatment by 2-factor ANOVA, $P < 0.05$. Values are means $\pm$ SE; $n$ = 5–7 in each experimental group.

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and RX rats (Fig. 1 and Table 1). The GSH concentrations in colonic mucosa and plasma were significantly correlated \((P < 0.005, \text{Fig. 1D})\).

Depletion of GSH with BSO results in Cys and CySS depletion in colonic mucosa and plasma and oxidation of Cys/CySS redox status in colonic mucosa. In vitro studies indicate that extracellular and intracellular Cys availability is an important regulator of protein synthesis and cell proliferation in a number of cell types (4, 27). Blockade of GSH synthesis by BSO may have a sparing effect on the GSH substrate Cys and theoretically result in increased intracellular Cys and CySS; however, previous results in in vitro systems are conflicting in this regard (4, 27). We examined Cys/CySS redox status in the colonic mucosa and plasma in our models in experiment 1 to explore this possibility. With BSO administration, colonic mucosal Cys concentration became markedly depleted in both TX and RX rats (Fig. 2A). Mucosal CySS remained unchanged (Fig. 2B), but Cys/CySS \(E_h\) became significantly oxidized in both

<table>
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<th>GSH*</th>
<th>GSSG*</th>
<th>GSH/GSSG (E_h)*</th>
<th>Cys*</th>
<th>CySS*</th>
<th>Cys/CySS (E_h)</th>
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<tr>
<td>TX/Sal</td>
<td>8.92 ± 0.81</td>
<td>0.97 ± 0.14</td>
<td>-141.74 ± 1.72</td>
<td>12.51 ± 0.05</td>
<td>40.02 ± 3.22</td>
<td>-87.55 ± 2.11</td>
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<tr>
<td>RX/Sal</td>
<td>8.41 ± 1.26</td>
<td>0.89 ± 0.33</td>
<td>-142.35 ± 2.53</td>
<td>13.67 ± 1.06</td>
<td>40.19 ± 1.75</td>
<td>-89.63 ± 1.88</td>
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<tr>
<td>TX/BSO</td>
<td>3.69 ± 0.75†</td>
<td>2.13 ± 0.97</td>
<td>-115.41 ± 10.50†</td>
<td>24.75 ± 6.37†</td>
<td>51.10 ± 6.28†</td>
<td>-98.59 ± 6.79</td>
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<tr>
<td>RX/BSO</td>
<td>5.07 ± 1.21</td>
<td>1.90 ± 0.82</td>
<td>-123.29 ± 13.02</td>
<td>16.01 ± 3.30</td>
<td>44.99 ± 2.96</td>
<td>-89.79 ± 6.29</td>
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Values are means ± SE; \(n = 5–7\) for each group. Plasma was obtained 7 days after small bowel resection (SBR) and 3 days after initiation of buthionine sulfoximine (BSO) or saline treatment (experiment 1). Thiols and disulfides were processed and analyzed by HPLC as described in MATERIALS AND METHODS. Redox potential \((E_h)\) is expressed as mV; plasma glutathione (GSH), glutathione disulfide (GSSG), cysteine (Cys), and cystine (CySS) are expressed in μM. TX, transection; RX, resection; Sal, saline. *Significant \((P < 0.05)\) main effect by 2-factor ANOVA for BSO to decrease plasma GSH, GSSG, Cys, and CySS concentrations and oxidize GSH/GSSG \(E_h\). †TX/BSO vs. TX/Sal, \(P < 0.05\).

Fig. 2. BSO treatment decreases colonic mucosal cysteine (Cys) concentrations and oxidizes colonic Cys/cystine (CySS) \(E_h\). See experiment 1 in MATERIALS AND METHODS for details of operations and treatment regimens. Cys and CySS concentrations were measured by HPLC, and Cys/CySS redox \((E_h)\) was calculated by the Nernst equation. A: colonic mucosal Cys concentration. B: colonic mucosal CySS concentration. C: mucosal Cys/CySS \(E_h\). D: correlation of colonic mucosal GSH concentration with Cys concentration. #TX/Sal vs. TX/BSO, \(P < 0.05\). †RX/Sal vs. RX/BSO, \(P < 0.05\). *Main effect of BSO by 2-factor ANOVA, \(P < 0.05\). Values are means ± SE; \(n = 5–7\) in each experimental group.
TX and RX groups (Fig. 2C). Two-factor ANOVA revealed a main effect for BSO to significantly decrease colonic mucosal Cys concentration and to oxidize Cys/CySS Eh in both TX and RX rats (Fig. 2, A and C). Thus GSH synthesis blockage resulted in depletion of the cellular Cys pool, with a highly significant positive correlation between colonic mucosal GSH and Cys concentrations (Fig. 2D). There was no correlation between plasma and colonic Cys (data not shown).

Our data showing that BSO decreases mucosal Cys is in keeping with previous publications, suggesting that GSH is involved in Cys uptake (6), transport, and storage (23, 31). In support of the role of GSH in Cys uptake, BSO administration induced a significant increase in plasma Cys and CySS concentrations (main effect by 2-factor ANOVA) that was most apparent in the SBR animals (P < 0.05 for Cys and CySS; Table 1). Plasma Cys/CySS Eh was not significantly altered by BSO.

Upregulation of colonic mucosal growth associated with BSO-induced oxidation of colonic mucosal GSH/GSSG redox status. Despite the marked depletion of the colonic GSH/GSSG and Cys/CySS pools by BSO, no morphological abnormalities were apparent in BSO-treated animals by light microscopy (Fig. 3A, top and middle). Electron microscopy of colonic sections showed intact colonic microvilli and no evidence of mitochondrial swelling or other indexes of cellular injury (Fig. 3A, bottom).

As shown in Fig. 3B, BSO treatment resulted in significantly increased colonic CD in both TX rats (+23%; TX/Sal vs.
TX/BSO; P < 0.05) and rats subjected to SBR (+30%; RX/Sal vs. RX/BSO; P < 0.05). Two-factor ANOVA revealed significant main effects of both SBR and BSO to increase CD without interaction. We estimated whether this growth response resulted from an increase in cell number (hyperplasia) or cell size (hypertrophy) by measuring the number of cells per crypt and number of cells per micrometer of crypt length. BSO induced a significant increase in the number of cells per crypt (main effect by 2-factor ANOVA) that was most apparent in the SBR animals (P < 0.05, Fig. 3C). In contrast, the number of cells per micrometer of crypt was unaffected by BSO (P > 0.05, Fig. 3D). These data indicate that BSO induced an increase in colonic crypt cell proliferation rather than merely an increase in cell size. This was confirmed by studies of BrdU incorporation in colonic crypt cells. The number of cells in the S phase of the cell cycle and thus incorporating BrdU increased by 48% (NS by 1-factor ANOVA) in TX rats and by 75% (P < 0.05 by 1-factor ANOVA) in RX rats with BSO treatment (Fig. 4A). Two-factor ANOVA revealed a main effect for BSO to significantly increase colonic BrdU incorporation in TX and RX rats (Fig. 4A). BrdU incorporation showed a slight increase in saline-treated rats (+9%; TX/Sal vs. RX/Sal; NS); but with BSO treatment, this increase was augmented and nearly achieved statistical significance (+29%; TX/BSO vs. RX/BSO; P = 0.06) (Fig. 4A). These changes occurred without a change in colonic crypt cell apoptosis in the study groups by both morphological criteria and by caspase-3/TUNEL staining (P > 0.05, Fig. 5).

Dose- and time-dependent effect of BSO-induced GSH depletion in promoting colonic mucosal growth. In experiment 1, stimulation of colonic mucosal growth occurred after BSO treatment, given from postoperative days 5 to 7, during which colonic mucosal GSH was depleted more than ~95% vs. controls. To further define this BSO modulatory effect, we conducted additional experiments with different BSO doses and periods of treatment. In experiment 2, rats in the RX/
BSOlow group exhibited a 70% GSH depletion in colonic mucosa and a more moderate oxidation of colonic GSH redox compared with the RX/BSO group (Fig. 6, A and B). Consistent with our data derived from rats studied in Experiment 1, the higher dose of BSO in Experiment 2 rats again led to a significant increase in colonic CD (~30%) and BrdU incorporation (~50%). However, the lower BSO dose did not induce any significant change in either of these colonic growth indexes (Fig. 6, C and D). The mucosal GSH level in the RX/Sal rats in Experiment 2 was higher than in Experiment 1, possibly due to the fact that different batches of animals were used. These data nonetheless support the concept that a threshold of GSH depletion is required to elicit the marked colonic growth effect observed in the 95% GSH depletion model.

In experiment 3, we started the BSO administration on the day of operation (with the same dosage as in experiment 1) and continued BSO treatment for 2 or 4 days postoperatively. These regimens resulted in a 79 and 87% reduction, respectively, in colonic mucosal GSH from controls (Fig. 7, A and B). On day 2 after the operation, colonic CD tended to be increased by BSO, and this response became statistically significant on day 4 (Fig. 7C).

**BSO administration increases colonic mucosal IGF-I mRNA expression.** Upregulation of endogenous mucosal growth factors, including IGF-I, may mediate adaptive colonic growth after partial small bowel colonic resection (7, 19, 36, 57). Therefore, we investigated the mRNA expression of IGF-I in colonic mucosa by real-time RT-PCR in experiment 2. Colonic mucosal IGF-I mRNA was modestly increased by SBR (~2-fold vs. control, RX/Sal vs. TX/Sal; NS). However, the low dose of BSO caused a further increase in colonic IGF-I mRNA expression (~3-fold vs. control, RX/BSOlow vs. TX/Sal; NS), and this was stimulated further at the high BSO dose (~4-fold vs. control, RX/BSO vs. TX/Sal; P < 0.05) (Fig. 8).

**DISCUSSION**

In this in vivo study, we observed that colonic epithelial cell growth and proliferation was enhanced and crypt cell apoptosis was unaffected when colonic mucosal GSH and Cys concent-
trations were markedly decreased by BSO with concomitant oxidation of the corresponding $E_h$ of the GSH/GSSG and Cys/CySS pools. Our observations are in contrast with in vitro data, indicating that cell growth is inhibited and cellular apoptosis enhanced by GSH depletion (5). In contrast, in studies in baby hamster kidney fibroblast (BHK-21) and human uterine cervical carcinoma (HeLa) cell lines, cellular levels of GSH declined during the growth phase (10). Furthermore, studies in the lung carcinoma cell line A549 (28) and in human aortic smooth muscle cells (9) demonstrated that upregulated growth occurred in the presence of BSO, consistent with our in vivo data. To our knowledge, our study is the first investigation to suggest that depletion of GSH and Cys upregulates tissue growth in vivo.

In the current study, we detected a main effect of SBR in increasing colonic CD in RX rats both with and without BSO administration. The increased growth response in RX/Sal vs. TX/Sal rats was not significant by one-factor ANOVA. However, SBR-induced colonic adaptive growth tended to be augmented in the presence of BSO. Previous studies have demonstrated modestly increased colonic proliferation, DNA and protein content, and CD in rat models of SBR with (36, 17, 18, 19) and without (42, 43, 52) partial colectomy. However, in our model of 80% jejunileal SBR, colonic adaptive growth appears to be limited (33, 36) compared with the adaptive growth in residual small bowel. Cell migration is one of the determinants of CD, in addition to the rates of cell proliferation and apoptosis (54, 57). Thus it is possible that the response to severe depletion of GSH in our study influenced cell migration and secondarily colonic CD. Determination of the effects of BSO/GSH depletion on colon crypt cell migration rates would be of interest.

The mechanisms in our study responsible for the association between oxidized redox status and colonic cell proliferation are unclear. However, several lines of evidence suggest that cellular GSH and Cys redox regulate cell growth and apoptosis by redox signaling (5, 26, 27). Numerous proteins with diverse functions in cell turnover are modified by $S$-glutathionylation, which may potentially stimulate or inhibit cell growth (20). In addition, the GSH/GSSG system interacts with other redox systems (ROS, Cys/CySS, and thioredoxin) in a complex redox signaling network (27). It has also been shown in vitro that BSO increases ROS (3, 14, 49, 50), which has been identified to regulate a host of signaling molecules (47) and stimulate cell

**Fig. 6.** Dose-dependent effect of BSO in promoting colonic mucosal growth. Rats were given different doses of BSO or saline during postoperative days 4–7 as described for experiment 2 in MATERIALS AND METHODS. Mucosal GSH concentrations (A) and mucosal GSH/GSSG $E_h$ (B) were determined by HPLC; crypt depth (C) was determined by light microscopy. The proliferation index (D) was determined by BrdU incorporation, as described in MATERIALS AND METHODS. RX/BSO$_{low}$ rats received a BSO dose resulting in $\sim$70% colonic mucosal GSH depletion compared with $\sim$95% GSH depletion in the RX/BSO rats. $\*P < 0.05$ vs. other groups. $n = 5–6$ in each group.
proliferation (8, 9, 24, 30). In a previous study with rat colonic epithelium, treatment with bile salt or xanthine oxidase resulted in increased ROS production in isolated cells and increased mucosal proliferation in vivo; both responses were abolished by superoxide dismutase (13). Because of the antagonistic nature of GSH and ROS, it is conceivable that ROS may have contributed to the BSO-induced growth response we observed.

IGF-I plays an important role in the regulation of intestinal growth and maturation (29, 34, 35). We previously showed that exogenous IGF-I increased colonic DNA synthesis and CD in an identical rat model of partial small bowel-colonic resection (36). We and others also reported that expression of IGF-I mRNA is upregulated in the colon at 1 wk after bowel resection in this model (19, 36). Given these data, we determined mRNA expression of IGF-I in rats from experiment 2.

The data shown in Fig. 8 demonstrate that IGF-I mRNA expression tended to increase with resection, was increased further with a low dose of BSO, and was significantly increased vs. control in the group that was administered a high dose of BSO, concomitant with an increase in colonic CD and an even more robust increase in BrdU incorporation in colonic crypts. The greater magnitude of increased BrdU incorporation compared with the increase in CD with a high dose of BSO can be explained by the fact that stimulated proliferation precedes changes in CD. The IGF-I gene expression data suggest that local generation of IGF-I may be involved in the mediation of colonic growth under conditions of severe GSH depletion in this rat gut adaptation model.

GSH concentrations in plasma and colonic mucosa were positively correlated, which is expected, as both are under the control of GCL. BSO induced a significant but less marked decrease in plasma GSH levels (59% decrease, Table 1) compared with colon values (95% decrease, Fig. 1). However, the control of plasma and tissue GSH is complex; plasma GSH levels are a function of the amount delivered to blood from body tissues and its clearance from the blood. Thus differences in the percent decrease in GSH levels in plasma and colon after BSO treatment are not unexpected.

An interorgan GSH-Cys cycle functions to homeostatically regulate Cys supply in tissues (44). The decreased colonic Cys levels and oxidation of Cys/CySS Eh in the present study after BSO treatment could reflect altered Cys transport and/or metabolism following BSO-induced changes in systemic GSH status. GSH facilitates cellular CySS uptake in rat hepatocytes by reducing it to Cys, which is more readily transported across cell membranes than CySS (6). Additionally, GSH also functions as a physiological reservoir for intracellular Cys and is considered an important moiety of Cys transport into cells (23, 31). The importance of GSH in cysteine delivery, uptake, and storage may explain the concomitant depletion of Cys that we observed in colonic mucosa with BSO, as evidenced by the positive correlation between colonic GSH and Cys concentrations. Our data are consistent with another study in which BSO decreased cellular Cys concentration in rat kidney (1). Plasma concentrations of Cys and CySS were increased in response to

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Fig. 7. Time-dependent stimulation in rats following 80% SBR. Rats were given BSO treatment, beginning on the day of operation, for 2 or 4 days as outlined in MATERIALS AND METHODS for experiment 3. Mucosal GSH concentration (A) and mucosal GSH/GSSG Eh (B) were determined by HPLC; crypt depth (C) was determined by light microscopy. ¶P < 0.05 vs. other groups with same period of BSO treatment. n = 2–5 in each group.

Fig. 8. BSO treatment increases IGF-I mRNA expression in colonic mucosa. Colonic mucosal samples were obtained from rats in experiment 2 and processed for real-time PCR expression of IGF-I mRNA as outlined in MATERIALS AND METHODS. IGF-I expression values are normalized by GAPDH mRNA and are expressed relative to TX/Sal; n = 3–4 in each group. #P < 0.05 vs. TX/Sal.
BSO in the current study (Table 1). This response was not likely due to increased liver GSH output (the major source of plasma Cys), given the BSO-induced blockade of GSH synthesis, or changes in food intake, given our successful pair-feeding regimen. The increase in plasma Cys and CySS may be due to decreased extraction of cyst(e)ine from blood, but confirmation would require studies of cyst(e)ine transport across tissues, including the gut. Plasma Cys/CySS $E_0$ remained unchanged after BSO administration. Thus changes in this parameter of extracellular redox status do not account for the colonic growth response in our models, as we have demonstrated previously with altered extracellular Cys/CySS redox in human cultured intestinal epithelial cells (25, 38, 41).

To our knowledge, the only previous study of gut mucosal morphology in response to BSO in vivo is the study of Martensson et al. (37) in intact mice. In contrast to the results of that study, we did not observe effects on crypt cell apoptosis or histological evidence of injury to colonic crypts, villi, or microvilli in our animals, despite marked BSO-induced GSH and GSSG depletion. Comparing the two studies, we depleted colonic mucosal GSH in our study to a similar extent (~95%) as Martensson et al., with a lower BSO dose over a shorter period of treatment (3 vs. 7 days). The different responses on colonic mucosal architecture between the two studies may be due to a longer period of BSO treatment in the study of Martensson et al., species differences, or differences in the colonic response to BSO in intact vs. small bowel-operated animals. In addition, higher doses of BSO, as used in the previous study (37), may induce unknown toxicities in addition to GSH depletion.

In summary, our data show for the first time that oxidation of colonic mucosal GSH/GSSG and Cys/CySS redox status stimulates colonic mucosal growth in vivo. This effect occurs in bowel-transsected rats and in rats after massive SBR, where the adaptive growth response is modestly enhanced. These effects were not mediated metabolically, i.e., through the sparing effect of BSO on the availability of Cys. In addition, a threshold effect for GSH depletion to induce colonic growth stimulation was observed, potentially mediated in part by upregulated local expression of IGF-I. We observed a colonic mucosal growth response when GSH was depleted in colon by 87–95% in two models of BSO administration, but this response was not significant with a lower level of GSH depletion (70%). Additional studies to determine the specific roles of redox-related components, including ROS, and the role of specific growth factors to stimulate colonic mucosal growth are needed to identify potential underlying mechanisms for these observations. Our data provide the first in vivo evidence suggesting that oxidized redox status in colon stimulates colonic mucosal cell growth. Our data also suggest that GSH is required to maintain normal colonic and plasma Cys/CySS homeostasis in rats. These findings may have translational relevance for conditions associated with either abnormal or adaptive stimulation of colonocyte proliferation.

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