Original Contribution

Marked changes in endogenous antioxidant expression precede vitamin A-, C-, and E-protectable, radiation-induced reductions in small intestinal nutrient transport

Marjolaine Roche a, Francis W. Kemp b, Amit Agrawal a, Alicia Attanasio a, Prasad V.S.V. Neti c, Roger W. Howell d, Ronaldo P. Ferraris a,b*

a Department of Pharmacology and Physiology, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, USA
b Department of Preventive Medicine and Community Health, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, USA
c Department of Radiology, New Jersey Medical School Cancer Center, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, USA

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A B S T R A C T

Rapidly proliferating epithelial crypt cells of the small intestine are susceptible to radiation-induced oxidative stress, yet there is a dearth of data linking this stress to expression of antioxidant enzymes and to alterations in intestinal nutrient absorption. We previously showed that 5–14 days after acute γ-irradiation, intestinal sugar absorption decreased without change in antioxidant enzyme expression. In the present study, we measured antioxidant mRNA and protein expression in mouse intestines taken at early times postirradiation. Observed changes in antioxidant expression are characterized by a rapid decrease within 1 h postirradiation, followed by dramatic upregulation within 4 h and then downregulation a few days later. The cell type and location expressing the greatest changes in levels of the oxidative stress marker 4HNE and of antioxidant enzymes are, respectively, epithelial cells responsible for nutrient absorption and the crypt region comprising mainly undifferentiated cells. Consumption of a cocktail of antioxidant vitamins A, C, and E, before irradiation, prevents reductions in transport of intestinal sugars, amino acids, bile acids, and peptides. Ingestion of antioxidants may blunt radiation-induced decreases in nutrient transport, perhaps by reducing acute oxidative stress in crypt cells, thereby allowing the small intestine to retain its absorptive function when those cells migrate to the villus days after the insult.

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The gastrointestinal (GI) tract is the second most radiosensitive critical organ system in the body, preceded only by the hematopoietic system. The continuously regenerating epithelial cells in the mucosal lining of the small intestinal lumen are readily perturbed by radiation [1,2]. These cells are located mainly in the crypt and crypt/villus junction of the intestinal mucosa. It is well established that γ-rays generate free radicals and reactive oxygen species (e.g., H2O2, e−, H2O−, OH•) through the radiolysis of water, the major constituent of tissue, thereby indirectly damaging DNA and other biomolecules.

The small intestine has four tissue types: epithelial, muscle, nervous, and connective tissue. Epithelial cells comprise the mucosal layer that lines the luminal surface. High levels of radiation exposure can cause intestinal epithelial denudation, and even higher doses can result in little or no subsequent regeneration, thereby affecting water, electrolyte, and nutrient uptake. Because the epithelial cells in the mucosal layer are responsible for digestive, absorptive, and barrier functions, acute radiation injury in humans contributes significantly to sugar malabsorption and diarrhea [3]. Secondary changes include infection, ulceration, and perforation. Early toxicity is mainly due to dysfunction of the mucosal epithelia [4]. Delayed or chronic radiation-induced toxicity typically involves all other tissue types and is a progressive condition with few therapeutic options and with substantial long-term morbidity and mortality. Severe delayed radiation enteritis is also serious, and about 10% of patients die as a direct result of radiation enteropathy. It is characterized by malabsorption, dysmotility, and transit abnormalities and features mucosal atrophy, intestinal fibrosis, and vascular sclerosis [4]. cDNA microarray analysis of the human ileum from patients with radiation enteritis revealed that genes involved in collagen synthesis, matrix metalloproteinase genes, and metalloproteinase inhibitor genes changed in expression, indicating morphological [5] changes being retained even several months after an acute 10-Gy insult. Early and delayed radiation enteritis were initially thought to be unrelated, but it is now known...
that the severity of early events may determine the subsequent incidence and severity of chronic enteritis [6].

We recently studied intestinal sugar transport before overt radiation-induced damage to the intestinal epithelium [7]. Here, the effect of ionizing radiation on nutrient absorption was delayed as demonstrated by reductions in mouse intestinal glucose and fructose transport about 5–14 days after acute whole-body γ-irradiation with 7–10 Gy. Transporter mRNA levels decreased in a dose-dependent manner, paralleling reductions in nutrient transport. Although the morphological and functional effects of radiation are well known, the response of the endogenous antioxidant network in the small intestine is poorly characterized and has not been correlated with physiological responses. Our previous attempts could not detect radiation-induced changes in antioxidant enzyme expression at the same time that sugar absorption rates decreased [7].

In the present study, we tested the general hypothesis that changes in the expression of antioxidant enzymes occur before overt changes in mucosal morphology and function. This was achieved by testing a variety of interrelated hypotheses: (i) radiation alters the expression of antioxidant enzymes within hours postirradiation, even though overt symptoms of malabsorption occur many days later; (ii) changes in antioxidant expression should be greater in the crypt region, where most differentiating intestinal cells are located; (iii) radiation-induced changes in the levels of 4-hydroxynonenal (4HNE; a biomarker of oxidative stress) [8] are greater in the intestinal epithelia than in other tissues of the small intestine, and they occur at crypt–villus locations, similar to adaptive increases in antioxidant enzymes; (iv) consumption of a cocktail of antioxidant vitamins A, C, and E should protect the small intestine from the harmful effects of irradiation. A cocktail of these vitamins was chosen based on their previously demonstrated radioprotective capacity. Studies in our laboratory showed that vitamin A substantially mitigates the deleterious effects of chronic exposure to both low- and high-LET radiations in mice testes [9]. Vitamin C mitigates radiation-induced damage even when administered after irradiation [10] and patients have benefited from taking vitamin C after abdominal radiotherapy [11]. Vitamin E has been reported to maintain jejunal, ileal, and colonic fluid absorption in irradiated rats [12]. Small intestinal crypt cell numbers, mucosal height, and goblet cell numbers were significantly protected from radiation effects by dietary vitamin E [13]. Cocktails of radioprotectors offer a means to further improve radioprotection. In fact, a combination of vitamins A, C, and E reduced bone marrow toxicity caused by radioimmunotherapy with 111In-labeled antibodies [14]. Vitamin C and E supplements reduced bleeding and diarrhea in patients with chronic radiation proctitis caused by pelvic irradiation [15]. Hence, vitamins A, C, and E have already been shown to protect the small intestine from radiation and therefore are excellent candidates for protecting against radiation-induced reductions in intestinal nutrient transport.

Materials and methods

Radiochemicals

glucose, 3-O-methyl-d-glucose, L-carnosine, and taurocholic acid were purchased from Sigma (St. Louis, MO, USA); D-fructose was from Mallinckrodt (St. Louis, MO, USA); and L-proline was from Eastman (Kingsport, TN, USA). D-[14C]Glucose (31 MBq/ml) and L-[3H]glucose (37 MBq/ml) were purchased from Sigma; D-[14C]fructose (19 MBq/ml), [3H]proline (37 MBq/ml), and [3H]taurocholic acid (37 MBq/ml) were from ARC (St. Louis, MO, USA); [3H]carnosine (37 MBq/ml) was from Moravek Biochemicals (Brea, CA, USA); and O-3-methyl-D-[14C]glucose (3.7 MBq/ml) and [carboxyl-14C]inulin (37 MBq/ml) were from PerkinElmer (Waltham, MA, USA).

Animals and irradiation

Swiss Webster adult male mice (average weight 37 g), 7–8 weeks of age, were purchased from Taconic Farms (Germantown, NY, USA). The experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School, University of Medicine and Dentistry of New Jersey (Newark, NJ, USA). Mice were housed, one per cage, in sterilized, filtered, positive-air-flow cages with feed and water provided ad libitum. Pelleted AIN-76A rodent diet (Research Diets, New Brunswick, NJ, USA) was used for the control diet (Table 1). Cages were maintained in a climate-controlled room with a 12-h light/dark cycle in our Cancer Center Comparative Medicine Resource Center. As detailed in our earlier study [7], whole-body radiation absorbed doses were delivered acutely with a Mark I 137Cs-irradiator (661 keV γ-rays) operating at a calibrated dose rate of 1.14 Gy/min (JL Shepherd, San Fernando, CA, USA). Sham-irradiated mice (0 Gy) were placed in the irradiator for the time equivalent to the maximum 137Cs source exposure period without activating the source. All irradiations were performed at room temperature.

Oxidative stress in irradiated animals

The study was divided into four trials done in series, with a total of 10–20 mice per trial (Fig. 1A). Mice were fed the control diet throughout the oxidative stress study. Whole-body doses of 8.5 Gy were delivered acutely and mice were randomly assigned to one of five groups. Groups were sacrificed at 1, 4, 8, 24, and 48 h postirradiation. Data from all trials were combined.

Protection against radiation-induced reductions in nutrient transport with a cocktail of vitamins A, C, and E

The study was divided into six separate, sequential but identical trials with six mice per trial (Fig. 1B). Data from all trials were combined. Mice were maintained as described above. A 2 × 3 factorial experimental design was followed with two diets and three radiation exposures. All irradiations were delivered acutely with a Mark I 137Cs-irradiator (661 keV γ-rays) operating at a calibrated dose rate of 1.14 Gy/min (JL Shepherd, San Fernando, CA, USA). Sham-irradiated mice (0 Gy) were placed in the irradiator for the time equivalent to the maximum 137Cs source exposure period without activating the source. All irradiations were performed at room temperature.

Table 1

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Acetate (250× control; Research Diets; Table 1). See the Discussion for the rationale of the dietary vitamin concentrations. This diet is referred to as the vitamin ACE-enriched diet.

All mice were acclimated for 10 days to the laboratory environment and the control rodent diet. After the 10-day acclimation period, the vitamin ACE-enriched diet was substituted for the control diet for the mice in the vitamin ACE group. This change provided a 4-day preirradiation ingestion of the vitamin-supplemented diet. Acute whole-body irradiation was carried out at absorbed doses of 8.5 or 10 Gy. Sham-irradiated mice (0 Gy) were placed in the irradiator, whole-body irradiation was carried out at absorbed doses of 8.5 or 10 Gy. Sham-irradiated mice (0 Gy) were placed in the irradiator, without activating the radiation source, for a duration equivalent to the time required to deliver the highest dose. Mouse weight and diet consumption were monitored before and after irradiation. The vitamin ACE-enriched diet was continued postirradiation until the animal was sacrificed.

**Animal surgery**

Mice were anesthetized with a cocktail containing 0.97% ketamine, 0.097% xylazine, 0.02% acepromazine in sterile water (5 ml/kg body wt). The small intestine was surgically removed 1, 4, 8, 24, or 48 h or 8 days postirradiation. The intestinal lumen was subsequently rinsed with an ice-cold Krebs Ringer bicarbonate solution (KRb1; 128 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 19 mM NaHCO₃, and 1.2 mM KH₂PO₄, pH 7.3–7.4) and immediately processed for determination of nutrient uptake and freezing for mRNA analyses, protein analyses, and sectioning for immunohistochemistry staining. After excision of the small intestine, the mouse was killed by intracardiac injection of 0.2 ml Euthasol (Virbac, Fort Worth, TX, USA).

**Intestinal uptake rates**

Uptake rates were determined in excised intestine as described earlier [7,16]. Briefly, several 1-cm segments of the duodenum and jejunum were individually mounted and everted on grooved steel rods (3-mm diameter). Because active transport of bile acids occurs only in the distal ileum, only this region was used for taurocholate uptake. Rods with the everted intestinal segment were preincubated at 37 °C for 5 min in KRb1 buffer and bubbled with 95% O₂–5% CO₂.

For glucose uptake, the tissue was incubated at 37 °C for 1 min in a freshly made oxygenated solution of KRb2 buffer (KRb1 buffer with 103 mM NaCl) containing 50 mM d-glucose, 380 μM [3H]glucose, and 0.2 μM L-[3H]glucose. Similarly, fructose uptake was measured after 2 min incubation in 50 mM D-fructose, 1.6 μM [14C]fructose, and 0.2 μM L-[3H]glucose. Uptake of the nonmetabolizable glucose analog 3-O-methyl-D-glucose was measured after 1 min incubation in 25 mM 3-O-methyl-D-glucose, 1.2 μM 3-O-methyl-[14C]glucose, and 0.2 μM L-[3H]glucose. L-[3H]Glucose was used to correct for adherent fluid and passive diffusion of glucose, fructose, and methyl-D-glucose. Finally, all sleeves were rinsed for 20 s in ice-cold KRb1 buffer with stirring.

For L-proline uptake, the segment was incubated in 50 mM L-proline, 0.2 μM [H]proline, and 20 μM [carboxyl-14C]luminol; l-carnosine in 25 mM l-carnosine, 0.9 μM [14C]l-carnosine and 20 μM [carboxyl-14C]luminol, and taurocholic acid in 0.65 mM taurocholic acid, 0.07 μM [14C]taurocholic acid, and 20 μM [carboxyl-14C]luminol. In all cases, [carboxyl-14C]luminol was used to correct for adherent fluid.

Each 1-cm piece of intestine was transferred to a preweighed glass vial, weighed, and dissolved in 1 ml of Solvable (PerkinElmer) at 37 °C overnight. After tissue digestion, a 10-ml volume of Ecolume (MP Biomedicals, Irvine, CA, USA) was added to each vial and vortexed briefly.

**mRNA analysis**

In the oxidative stress experiment, a 2-cm piece of jejunum was scraped to remove the mucosa, which was then immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcriptase PCR using an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) and a SuperScript III reverse transcriptase kit (Invitrogen) according to the manufacturer’s instructions. The cDNA obtained was subjected to real-time PCR for the SOD1, SOD2, catalase, glutathione peroxidase 1, and β-actin genes using the Mx3000P QPCR System (Stratagene, La Jolla, CA, USA). The real-time PCR was performed with Brilliant SYBR green QPCR master mix reagent (Stratagene). Earlier work showed β-actin expression to remain unaltered within the range of radiation doses used in this study, thereby serving as our housekeeping gene [7]. Thermal cycling proceeded with 40 cycles (initial activation step 10 min at 95 °C; 40 cycles of 30 s at 95 °C, 1 min annealing temperature of primers, 30 s at 72 °C; and terminal step 1 min at 95 °C and 30 s at 55 °C). The amount of RNA was calculated with relative standard curves for RNA of the specific genes studied. Normalization to β-actin was conducted to account for variability in the concentration of total RNA. To examine for genomic contamination, the reaction was also carried out in samples that did not receive reverse transcriptase amplification reagents. A reverse transcriptase PCR was then carried out on total RNA, followed by three determinations by real-time PCR with the following primer sequences:

1. SOD1 (forward 5’-CAACCGGTGAACAGTGTTGTTGT-3’, reverse 5’-TCACATTGCCAGCTCCTGCTCACC-3’); SOD2 (forward 5’-
TGGAGACCCAAAGGAGGTGTCT-3′; reverse 5′-TGTGTTCTCTGG- AATGGTCTTGG-3′); (2) catalase (forward 5′-AGACAAAGGCTTGTTGTA- GAACA-3′, reverse 5′-AGTCAGGTGGACGTACGAAAT-3′); and (3) glutathione peroxidase 1 (forward 5′-TTTCCGTCACTGAGTTCG- GACA-3′, reverse 5′-AGCCCTCTACACCCACAGCA-3′); and (4) β-actin (forward 5′-TGTATCACACTGGAGCAGCATG-3′, reverse 5′-CTGGGGTGTTGAAGGTCTCAAACA-3′). These primers were designed to amplify only the mRNAs.

Western blot

A 2-cm piece was excised from the jejunum and scraped to separate the mucosa from muscle tissue, and the mucosa was immediately frozen in liquid nitrogen and stored at −80 °C. Frozen tissue (~100 mg) was homogenized in 1 ml of T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA) with 10 μl of complete protease inhibitor cocktail (Roche, Mannheim, Germany), incubated on ice (10 min), and centrifuged (20 min) at 12,000 rpm at 4 °C. The supernatant was kept for protein quantification by the Bicinchoninic Acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA). Samples were prepared with a Lane Marker Reducing Sample Buffer 5× (Thermo Scientific) by boiling for 7 min. Samples and ladder were loaded onto a 0.45-μm pore nitrocellulose membrane (Millipore, Billerica, MA, USA) using a Mini Trans-Blot Cell (Bio-Rad). The membrane was incubated in 5% nonfat milk in Tris-buffered saline–Tween 20 followed by overnight incubation with a primary antibody (4 °C). After being washed, the membrane was incubated for 1 h with the secondary antibody. The primary antibodies were rabbit anti-mouse superoxide dismutase 1 (Millipore), rabbit anti-mouse superoxide dismutase 2 (Millipore), and goat anti-mouse catalase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). We were unable to find an anti-glutathione peroxidase 1 antibody that worked. The secondary antibodies were all horseradish peroxidase-conjugated antibodies: donkey anti-rabbit antibody (Jackson Immuno Research, West Grove, PA), sheep anti-mouse antibodies (GE Healthcare, Piscataway, NJ, USA), and rabbit anti-goat antibodies (Millipore). Horseradish peroxidase was developed using a Western blotting detection kit (GE Healthcare) and autoradiography film (Denville Scientific, Metuchen, NJ, USA).

Preparation of intestinal mucosa for immunohistochemistry staining

Intestinal tissue (0.5 cm long) was fixed in 4% buffered paraformaldehyde, embedded in paraffin, and sectioned (6 μm thick). Sections were deparaffinized and rehydrated, and epitope retrieval was achieved in 10 mM citrate buffer (pH 6.0) in an autoclave. After initial incubation in 1% (for SOD1 sections) or 5% (for 4HNE sections) normal goat serum in PBS, sections were subsequently exposed to the primary antibody overnight at 4 °C.

Although there are no specific biomarkers for radiation-induced damage, oxidative stress arising from it has been detected by monitoring changes in levels of antioxidants, antioxidant enzymes, and biomarkers of oxidative stress such as 4HNE. 4HNE is a toxic unsaturated aldehyde formed during lipid peroxidation that can also bind to proteins to form carbonyls [8]. Thus we tracked the location of tissues that experienced radiation-induced oxidative stress with 4HNE.

Primary antibodies were rabbit anti-mouse SOD1 (1:1000 dilution in PBS) or rabbit anti-4HNE (1:200 dilution in PBS). Slides were rinsed with PBS and then incubated with a secondary antibody (Cy3-conjugated rabbit anti-goat IgG (1:200) or Alexa 488-conjugated goat anti-rabbit (1:200)). Sytox green nucleic acid stain (1:300 dilution with deionized water) was applied to SOD1 sections and incubated for 15 min, followed by rinsing with distilled water. Coverslips were mounted to each slide using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Similar slides were prepared with the substitution of PBS for the primary antibody to check for nonspecific binding and extraneous excitation of the secondary antibody. Processing of tissues from different treatments was done using, where applicable, exactly the same solutions. Slides were viewed through a Zeiss LSM510 confocal microscope with argon laser (458, 488, 514 nm) and HeNe laser (543 nm) for simultaneous collection of two fluorescent signals. To facilitate comparison of images, acquisition of images was accomplished using exactly the same confocal settings (see Supplemental Fig. S3).

Statistics

Values are expressed as means ± standard error. Two-way ANOVA or general linear model (GLM) analysis for unbalanced data was used to determine the statistical significance. Pairwise differences were evaluated subsequent to two-way ANOVA or GLM data by comparison of least-square means. Statistical evaluations were performed using StatView 5.0 (Abacus Concepts, Berkeley, CA, USA) and SAS 9.2 (SAS Institute, Cary, NC, USA).

Results

Nutrient uptake at 24 and 48 h postirradiation

We have previously shown that radiation-induced decreases in intestinal sugar uptake and transporter mRNA levels were observed mainly 8–15 days after acute whole-body γ-irradiation with absorbed doses of 7–10 Gy [7]. We now find that there was little or no change in antioxidant mRNA expression in the intestinal mucosa during this time (Supplemental Fig. S1). Conversely, to demonstrate that the intestine was absorbing nutrients within 48 h postirradiation, when most changes in antioxidant expression described below occurred, we show that glucose uptake did not change significantly with dose or time postirradiation (Fig. 2A). Fructose, proline, and carnosine uptake (Figs. 2B–D) also did not change significantly with radiation dose (P>0.80 by two-way ANOVA for all nutrients) and time postirradiation (P>0.17 for all nutrients).

Time course of changes in mRNA expression of antioxidant enzymes

In contrast to the absence of radiation-induced reduction of nutrient transport at t<48 h, levels of the cystolic antioxidant enzyme
SOD1 mRNA varied with postirradiation time \((P=0.02\) by two-way ANOVA\) and dose \((P=0.06)\), with a highly significant interaction between postirradiation time and dose \((P=0.01; \text{Fig. } 3A)\). The statistically borderline dose dependence is based on the fact that mRNA abundance was not expected to and actually did not change significantly in unirradiated mice. The significant interaction indicated that the dependence of mRNA abundance on dose depended on postirradiation time \((\text{Fig. } 3A)\). For example, SOD1 expression was similar for 0 and 8.5 Gy at both 4 and 48 h postirradiation. At 8 and 24 h, mucosal SOD1 mRNA abundance was two to four times greater in 8.5 Gy mice compared to the abundance in control mice sacrificed at the same time. In contrast, at 1 h postirradiation, the SOD1 mRNA abundance in unirradiated mice was several fold greater than in irradiated mice. It should be noted that because the RNA was extracted only from the mucosa, these radiation-induced changes reflected changes mainly in the intestinal absorptive cells.

Patterns of expression were similar for the other antioxidant enzymes. For mRNA levels of mitochondrial SOD2, their dependence on postirradiation time \((P=0.11)\) and dose \((P=0.06)\) was borderline but the interaction was quite robust \((P=0.01; \text{Fig. } 3B)\). For SOD2, differences were observed mainly at 4, 8, and 24 h, when expression increased by two to three times in irradiated mice. The mRNA abundance of catalase varied with postirradiation time \((P=0.03)\) but not dose \((P=0.22)\), and the interaction of these two variables was highly significant \((P=0.0007; \text{Fig. } 3C)\). Catalase mRNA levels were greatest at 24 h postirradiation and decreased markedly by 48 h to levels observed at 1 h postirradiation. For glutathione peroxidase 1 \((\text{Fig. } 3D)\), mRNA levels depended significantly on postirradiation time \((P=0.007)\) and dose \((P=0.002)\), and there was an interaction between dose and postirradiation time \((P=0.0005)\). Glutathione peroxidase 1 mRNA abundance peaked at 8 h to levels six times greater than control and then decreased gradually.

It is important to point out that mRNA levels of all antioxidant enzymes in unirradiated mice did not vary as a function of time \((P>0.50\) by one-way ANOVA\). This strongly supports consistency in sampling and analysis. Moreover, the levels of mRNA for all antioxidant enzymes in irradiated mice were always much less \((3–8\times \text{ decrease}, P<0.02)\) than in controls at 1 h. This indicates that the effect of acute whole-body irradiation on antioxidant enzyme expression was triphasic: (i) rapid decrease of steady-state levels of mRNA occurring in minutes, (ii) dramatic upregulation occurring within hours, and (iii) another downregulation occurring within a day or two after irradiation. Expression of antioxidant mRNA in irradiated mice was \(10–20\times\) less at 1 h compared to that at peak expression occurring 7–23 h later. Finally, like the findings at 48 h, it was determined that at 8 days postirradiation, mRNA expression levels of SOD1, SOD2, catalase, and glutathione peroxidase 1 were similar in irradiated and unirradiated mice (Supplemental Fig. S1).

**Abundance of antioxidant enzymes after acute irradiation**

The pattern of radiation-induced changes in protein abundance of antioxidant enzymes was less pronounced but generally followed that for mRNA abundance \((\text{Fig. } 4A)\). Like their mRNA abundance, intestinal SOD1, SOD2, and catalase protein levels in irradiated mice tended to peak 8–24 h postirradiation \((P=0.10\) by one-way ANOVA\), but generally by only about twofold compared to unirradiated mice \((\text{Fig. } 4B)\). Because the protein homogenates were derived from mucosal scrapes, these changes reflect alterations in levels of antioxidant enzymes in the absorptive cells.

**Levels and immunolocalization of 4HNE and SOD1**

Levels of 4HNE were apparently greater in the small intestine of mice 8 h after acute whole-body irradiation with 8.5 Gy \((\text{Fig. } 5)\). The 8-h time point was chosen because the greatest antioxidant enzyme response was observed at this time postirradiation. In unirradiated mice, there was little fluorescence evident in the absence of primary antibody \((\text{Fig. } 5A)\), and there was little evidence of endogenous 4HNE when probed with anti-4HNE \((\text{Fig. } 5B)\). Absence of primary antibody also produced little fluorescence in the intestine of irradiated mice \((\text{Fig. } 5C)\). Levels of 4HNE were much greater in epithelial cells (e) lining the intestinal lumen as opposed to the submucosa (s) and muscle (m) layers underneath \((\text{Fig. } 5D)\). Even when irradiated, there was negligible fluorescence with no antibodies or with only the primary antibody, thereby suggesting no endogenous fluorescence \((\text{Supplemental Figs. } S2A\text{ and } S2C)\). Different sets of unirradiated and irradiated mice showed similar results \((\text{Supplemental Figs. } S2B\text{ and } S2D)\).

Increases in the levels of SOD1 seemed much greater in the intestinal epithelial cells relative to the submucosa and muscle layers \((\text{Fig. } 6)\). Intestines of unirradiated mice had low levels of SOD1 \((\text{Figs. } 6A–D)\), with greatest concentrations in the crypt regions. Constitutive SOD1 expression seems to be mainly in the cytosol and appears to be higher in the crypt region (c), where cells are rapidly dividing, compared to upper villus regions (v). There is almost no fluorescence in the absence of SOD1 antibody \((\text{Figs. } 6E–H)\). Intestines of mice 8 h after irradiation with 8.5 Gy seemed to show increases in levels of SOD1 \((\text{Figs. } 6I–L)\)—these results confirm findings of Western blots \((\text{Fig. } 4)\) obtained from different mice, which indicated greater SOD1 expression in irradiated mice. There was little SOD1 expression in the basal lamina right below the crypt. Most increases in SOD1 expression appeared to be in the crypt region \((\text{Supplemental Fig. } S3)\). In the absence of SOD1 antibody, there was little fluorescence \((\text{Figs. } 6M–P)\) in intestinal sections obtained from irradiated mice.

The villus–crypt difference in fluorescence is markedly greater when the antibody is present than when it is absent \((\text{compare Figs. } 6L\text{ and } D\text{ to } P\text{ and } H)\), suggesting that the radiation-induced villus–crypt gradient in fluorescence without the antibody cannot fully explain the marked, radiation-induced difference in the villus–crypt fluorescence gradient with the antibody. Some compounds such as NADH exhibit autofluorescence \([17]\), and radiation-induced perturbations in the levels of

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**Fig. 3.** Relative mRNA abundance of antioxidant enzymes in isolated jejunum of unirradiated \((\text{open bars})\) and irradiated \((8.5\text{ Gy, filled bars})\) mice. (A) SOD1, (B) SOD2, (C) catalase, and (D) glutathione peroxidase 1 as measured by real-time PCR. mRNA was collected from the mucosa of the proximal small intestine of mice fed control diet and sacrificed 1, 4, 8, 24, and 48 h postirradiation. Results are means \(\pm SE\) \((n=6)\). Filled bars with different superscripts are significantly different \((P<0.05\) by one-way ANOVA\). Thus, there is a marked decrease in the levels of mRNA at 1 h postirradiation, followed by a dramatic increase, and then a decline to levels similar to those of unirradiated mice. Open bars are similar throughout, indicating that expression in unirradiated mice did not change over time.
these autofluorescent compounds in the crypt may be the source of the small villus–crypt gradient in endogenous autofluorescence shown in Fig. 6P. Although there was little change in the intensity of nuclear staining upon irradiation (Figs. 6A, E, I, M), there were marked radiation-related changes in the intensity of SOD1 fluorescence (Figs. 6B, F, J, N and D, H, L, P). SOD1 levels in epithelial cells seemed greater than those in the submucosa and muscle (Figs. 6D and L).

**Radioprotection of intestinal nutrient transport**

**Dietary intake, body weight, and intestinal weight**

Consumption of the vitamin ACE diet was not significantly different from that of the control diet (Fig. 7). However, at 8 days postirradiation, food intake in the 10-Gy mice was reduced by ~25% (P = 0.0001) in comparison to both the 0- and the 8.5-Gy-exposed mice, whose food intakes did not differ or differed modestly. Our earlier studies in calorie-restricted mice indicate that acute (2-day to 2-week) reductions in daily food consumption by up to 30% have no effect on intestinal nutrient transport [18]. Body weights were independent of dose except at 8 days postirradiation (P = 0.03). Total intestinal weight is largely unchanged 8 days after irradiation with 8.5 Gy (Table 2). However, irradiation with 10 Gy results in a small but significant (P < 0.05) reduction in total intestinal weight. This reduction is mitigated significantly by the vitamin ACE diet (Table 2).

**Sugar transport**

To clearly demonstrate protection, we first tested by one-way ANOVA if radiation reduced intestinal uptake of sugars in mice fed the control diet and, if it did, whether a cocktail of the dietary vitamins A, C, and E prevented radiation-induced decreases in uptake. D-Glucose uptake decreased with dose in control (P = 0.05) but not in vitamin-supplemented (P = 0.80) mice (Fig. 8A). Uptake of the nonmetabolizable analog 3-O-methyl-D-glucose tended to decrease with dose (P = 0.20), but not when mice were consuming vitamins A, C, and E (P = 0.85; Fig. 8B). The 30% decrease in D-glucose and 3-O-methyl-D-glucose uptake at 10 Gy was clearly prevented by the antioxidant vitamins. Fructose uptake decreased with dose by threefold...
Fructose uptake also decreased in irradiated mice that were fed vitamin-supplemented diets ($P = 0.02$); however, at 10 Gy, fructose uptake in vitamin-supplemented mice was much greater (threefold) than in unsupplemented mice. Likewise, D-glucose ($P = 0.04$) and 3-O-methyl-D-glucose ($P = 0.09$) uptake was also greater in vitamin-treated mice than in control mice at 10 Gy. Treatment with vitamins also tended to rescue D-glucose uptake at 8.5 Gy ($P = 0.17$).

**Amino acid, peptide, and bile acid transport**

Intestinal uptake of nutrients other than sugars also decreased with dose. In mice fed control diets, proline absorption decreased in a dose-dependent manner, with 30 and 40% reductions at 8.5 and 10 Gy, respectively ($P = 0.008$; Fig. 8D). Supplemeting the diet with vitamins ACE again prevented the decrease ($P = 0.68$). Absorption of the dipeptide carnosine also decreased with dose in dietary control ($P = 0.04$) but not in vitamin-supplemented mice ($P = 0.95$) (Fig. 8E). Taurocholate absorption was independent of radiation dose in mice eating either the control ($P = 0.24$) or the vitamin-supplemented ($P = 0.23$) diet (Fig. 8F). In all cases, patterns of nutrient uptake, when expressed per milligram (not shown), mirrored those expressed per centimeter of intestine.

At 10 Gy, proline, carnosine, and taurocholate uptake was greater ($P = 0.01$ to 0.07) in vitamin-treated than in control mice, suggesting that antioxidant vitamins increased uptake rates at absorbed doses that compromised nutrient transport.

**Total glucose uptake**

To portray the effect of acute $\gamma$-irradiation on nutrient absorptive function of the entire intestine, glucose uptake was determined not only in the jejunal (Fig. 8A) but also in the duodenal and ileal regions of the small intestine (not shown). When uptake was integrated from proximal through distal regions, the total uptake capacity of the intestine could be estimated (Fig. 9). Total absorptive capacity of the intestine for glucose decreased with dose ($P < 0.05$), but not in mice fed the vitamin ACE diet ($P = 0.50$).

**Intestinal permeability**

D-Glucose uptake, a measure of intestinal permeability, was affected neither by diet nor by radiation (Supplemental Fig. S4).
Summary of nutrient uptake studies

In summary, 8 days after acute irradiation with 8.5 and 10 Gy, intestinal absorption of most nutrients decreased, but consumption of antioxidant vitamins prevented these radiation-induced decreases in nutrient uptake.

Discussion

About 600,000 cancer patients undergo radiation therapy each year, of which over 200,000 involve the abdominal and pelvic areas. Intestinal dysfunction occurs in 60–90% of patients receiving abdominal radiation therapy, and a conservative estimate of the number of patients with radiation-induced intestinal dysfunction living in the United States is 2 million [4]. Prognosis is poor, and some patients require prolonged parenteral nutrition and about 10% eventually succumb. The side effects of irradiation on intestinal structure and function, termed radiation enteritis, dictate the upper limits of radiation therapy involving abdominal organs. Although radiation enteritis is a severe problem in patients receiving abdominal or pelvic radiation therapy, there is no standardized strategy for medical pre-

vention and therapy [19]. A major gap in knowledge pertaining to radiation enteritis is the absence of studies linking changes in oxidative stress and adaptations of the antioxidant network with alterations in intestinal function. Additional information about this link may shed light on methods that may increase the radiation tolerance of normal intestinal tissue to improve the quality of life of cancer survivors and to enhance radiation efficacy by dose escalation [20].

The main and novel findings of this study are that (1) the time course of radiation-induced changes in the expression of antioxidant enzymes differs from that of radiation-induced reductions in intestinal absorptive function, (2) the cell type and location expressing the greatest changes in levels of oxidative stress and in antioxidant enzymes are, respectively, epithelial cells responsible for nutrient absorption and the crypt region comprising mainly undifferentiated cells, and (3) a cocktail of the antioxidant vitamins A, C, and E is an effective radioprotector of intestinal absorptive function when consumed before irradiation. Because nutrient transporters are expressed only in epithelial cells, the tissue type with the greatest levels of 4HNE, it is not surprising to detect reductions in nutrient uptake several days after intestines are exposed to radiation. Two minor findings reported here for the first time are that (1) the radiation-induced decline in glucose transport is probably due to effects on the transport step, because radiation also decreased the transport of the nonmetabolizable glucose analog, 3-O-methyl-glucose, and (2) the absorption of the dipeptide carnosine decreased as a function of radiation absorbed dose.

Oxidative stress and antioxidant enzymes in intestinal mucosa

There are only a few studies that have focused on understanding radiation-induced oxidative stress on the small intestine. Repeated low-dose exposure (0.25 Gy monthly irradiation for a total of 4.5 Gy) of the whole body increases the concentration of thiobarbituric acid-reactive substances (a biomarker of oxidative stress) and activities of glutathione peroxidase in the small intestine but not in most other organs [21], indicating the highly radiosensitive nature of the small intestine relative to other organ systems.

Radiation-induced changes were observed in small intestinal mRNA and protein expression, as well as in the activity of several antioxidant enzymes, in antibiotic-treated mice exposed to high dose (14 Gy) abdominal irradiation [22]. Of the nine antioxidant enzymes examined, only SOD2 and thioredoxin 2 (both localized in the mitochondria) responded 6 h after exposure of the small intestine to 14 Gy. These interesting findings were similar to our results and provide additional evidence of the rapid response of intestinal antioxidant enzymes to acute high-dose irradiation. No other time points were examined by Haton et al. [22]. However, it may be essential to track the time course of the antioxidant response to irradiation, because the magnitude of change in mRNA expression of these enzymes may be transient and seems dependent on time after irradiation. In mouse skin, the redox-sensitive transcription factor NF-κB and a group of NF-κB-related proteins are activated within 15 min of exposure to ionizing radiation [23], leading to increases in SOD2 expression and activity 24 h later [24]. Thus, it will be interesting to determine whether NF-κB inhibitors will prevent the rapid and transient radiation-induced increases in expression of antioxidant enzymes in mouse small intestine in vivo.

Four days after acute abdominal irradiation of mice with 14 Gy, intestinal structural damage was evident, and it was paralleled by reductions in SOD1, SOD2, and catalase mRNA expression and activity [22]. These findings are not surprising because anatomical destruction of the intestinal tissue would reduce if not outright prevent the expression of many genes and probably indicate that the antioxidant network of the intestine has been overwhelmed. We have earlier shown, by tracking nutrient uptake for several days after irradiation,
that at 8.5–10 Gy, nutrient absorption decreases ~8–14 days after irradiation [7]. Reductions in absorptive function decrease before overt morphological damage can be observed.

Other in vivo studies have used the approach of providing antioxidant supplements, as well as potentially radioprotective compounds, and then evaluating radiation-induced changes in the levels of antioxidants and antioxidant enzymes in animal models. In these studies, lipid peroxides have generally been shown to increase with absorbed dose. However, it was not consistently clear from these studies whether irradiation increases endogenous antioxidant enzyme expression or activity and whether glutamine in humans [25], selenium in rats [26], vitamin E in rats [26], Triphala in mice [27], and melatonin in rats [28] could enhance endogenous antioxidant activity in the small intestine after irradiation. Perhaps the failure to observe clear effects of exogenous antioxidants on endogenous antioxidant enzyme expression or activity is due to the transient nature of the response of endogenous antioxidants. Regardless of their inconsistent effect on endogenous antioxidant expression or activity, these exogenous antioxidants were consistently shown to offer radioprotection for the small intestine by reducing levels of oxidative damage.

**Location of radiation-induced antioxidant enzyme expression**

The crypt–villus site of radiation-induced changes in intestinal antioxidant enzyme expression is apparently unknown, even though this information is critical because it identifies the cell types or locations that respond to radiation insults regardless of whether other overt changes are apparent. Here we show that, although radiation-induced increases in 4HNE levels are remarkable in almost all tissue types, the greatest increase seems localized mainly in the innermost mucosal layer comprising epithelial cells. Because 4HNE levels seem homogeneous along the crypt–villus axis of epithelial cells, oxidative damage (as indicated by 4HNE location) may be similar among epithelial cells at various stages of development. In contrast, the crypt–villus location of antioxidant enzyme adaptation clearly indicates that SOD1 expression first increases markedly in crypt cells. Other epithelial cells along the crypt–villus axis differ not only in terms of development, but also in terms of radiosensitivity [1,29], with differentiated villus cells being relatively much less radiosensitive. Actively proliferating sensitive cells in the body, and their sensitivity (LD50 ~ 20 Gy [32]).

**Intestinal irradiation slows down the rate of mitosis and proliferative activity of crypt cells and therefore the replacement of villus cells within 12–48 h postirradiation [29,33]. Replacement of villus cells is dependent on the number of and rate of division of surviving stem cells in the crypt. If no crypt cells survive at very high doses, then the villus is denuded and ulcers occur and nutrient transport**
decreases without recovering. If some crypt cells survive, the villus is eventually regenerated and the time of regeneration is dependent on the severity of the insult to the crypt. When the villus is repopulated with functionally mature enterocytes, nutrient transport recovers.

There have been reports that microvascular endothelial apoptosis in the submucosal and muscle layers is the primary lesion leading to intestinal dysfunction [31]. However, at absorbed doses above and below the threshold for death from the GI syndrome, recent work has demonstrated that vascular endothelial cell apoptosis does not seem to be the cause of the GI syndrome [34]. Most cells affected by ionizing radiation actually reside in the epithelium.

The gastrointestinal glutathione peroxidase 2 is highly expressed in the intestinal crypt region [35], and it will be interesting to evaluate its role in radioprotection, because irradiation seems to markedly affect the crypt region. Glutathione peroxidase 2 is protective against intestinal inflammation in vivo [36] and its expression, as well as activity, is induced by intestinal luminal microflora [37].

**Radioprotection of intestinal transport function**

Although there has been a large number of studies evaluating potential radioprotectors for the small intestine, there has been only a few that focused on radioprotection for nutrient absorptive function. When given before irradiation, a diet high in saturated, but not polyunsaturated, fatty acids prevented the radiation-associated decline in intestinal uptake of glucose and some fatty acids [38]. A synthetic prostaglandin (enprostil) did not prevent the radiation-associated decline in intestinal glucose uptake when given before or after irradiation [39]. Preradiation treatment with antioxidant selenium led to a lower incidence of diarrhea in patients receiving pelvic radiation [40], suggesting that consumption of antioxidants before irradiation can diminish the adverse effects of radiation on intestinal function.

The top five nutraceuticals in terms of U.S. sales are vitamin C, coenzyme Q, vitamin E, vitamin A\(\alpha\)-carotene, and nontoxic. We have previously shown that vitamin A alone does not protect the small intestine from damage caused by acute whole-body irradiation [7], hence we here chose to provide a cocktail of vitamins A, C, and E, a mixture that turned out to be an excellent radioprotector. The experimental level of 0.3 g or 150,000 IU/kg diet of vitamin A has been shown by Friedenthal et al. to be highly protective of the GI tract against radiation damage [41]. This level is nontoxic to rats even when consumed for 1 year [42]. Humans consuming 1,000,000 IU/day for 5 years have not experienced vitamin A toxicity [43]. About 100× the RDA is the likely dietary limit [44].

The control diet did not contain vitamin C, which mice synthesize. The optimal level of vitamin C for radioprotection of testes is about 10 g/kg diet [45], hence we added an equivalent (in terms of activity) amount (29 g/kg diet) of vitamin C phosphate (only 35% active). When injected into target tissue or peritoneum, vitamin E is a highly effective radioprotector particularly for the small intestine [26,46], but injections are often impractical. Vitamin E may be added at 3× the minimum required in the diet to be effective in intestinal and hepatic radioprotection [26,47]. Dietary vitamin E, at 30× control values, suppresses lipid peroxidation in cancerous tissue [48]. Even higher levels (250×, 10,000 IU/kg diet) protected crypt cell numbers, mucosal height, and goblet cell numbers against radiation effects [13]. Although very high, these levels consumed for 2 weeks did not cause vitamin E toxicity in rats [13].

Dietary antioxidant vitamins can potentially protect both normal and malignant gut tissues from irradiation. Thus, patients who take antioxidant vitamins during radiation therapy of their malignant tumor may have fewer bouts of diarrhea, but may also experience reductions in the anti-tumor efficacy of ionizing radiation. However, Blumenthal et al. have shown that, in the context of radioimmunotherapy of GW-39 human colon cancer xenografts in mice with 131I-MN14 anti-CEA IgG, a cocktail of vitamins A, C, and E facilitated a 42% increase in maximum tolerated dose and protected against reductions in body weight and myelosuppression [14]. They also observed that, although this cocktail of vitamins afforded considerable protection of normal tissues, no protection was extended to the tumor. These findings are consistent with recent epidemiological studies conducted by Simone et al. that show that patients who underwent radiation therapy and took nonprescription antioxidants actually had increased survival [49,50]. Taken together, these and our studies auger well for the use of vitamins as radioprotectors of normal tissues in the context of radiotherapy of cancer.

**Perspective**

In summary, using radiation absorbed doses that clearly result in marked reductions in nutrient transport and that acutely increase levels of oxidative stress and expression of antioxidant enzymes in intestinal epithelial cells responsible for nutrient absorption, we found that ingestion of high levels of antioxidants for a few days before irradiation may blunt radiation-induced oxidative stress and allow epithelial cells of the small intestine to retain its absorptive function. Although the implications of these findings to radiation oncology were already alluded to in the first paragraph of the Discussion, they also have implications for radiological emergencies or terrorism. The GI injuries that arise in casualties from such radiation exposures can lead to temporary debilitation or death. Although death is catastrophic indeed, the economic and social implications of temporary debilitation can also be enormous. Accordingly, development of medical products capable of ameliorating GI damage caused not only by otherwise lethal radiation exposures, but also by sublethal exposures that may adversely impact important GI functions such as nutrient transport, is necessary. In this way, one can reduce the debilitating effects of sublethal doses of radiation to keep the general population maximally productive under emergency conditions.

Specialized agents can be manufactured and stockpiled for use by emergency responders; however, making a fresh supply of such agents immediately available to the potentially large number of victims in the general public is complex. Therefore, identifying radioprotectors that are readily available to the general population, without the need for stockpiling, is desirable. The chemotoxicity of the most effective synthetic radioprotectors often limits their use in humans [51]. Accordingly, Weiss and Landauer [52] have suggested the use of naturally occurring antioxidants and related agents. The present study provides support for this premise and argue in favor of using a combination of vitamins A, C, and E to protect nutrient transport against radiation-induced intestinal damage caused by accidents or terrorist events. These vitamins are inexpensively manufactured, easily administered, safe for repeated doses, relatively stable, and readily available to the general public. Additional studies are under way to assess the capacity of this cocktail to protect intestinal transport against chronic irradiation and to ameliorate reductions in transport when administered after the advent of an acute or chronic radiation insult. It is recommended that the consequences of intake of high doses of this vitamin cocktail be studied in humans.

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