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Palmitoylethanolamide regulates development of intestinal radiation injury in a mast cell dependent manner

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Abstract

Background—Mast cells and neuroimmune interactions regulate the severity of intestinal radiation mucositis, a dose-limiting toxicity during radiation therapy of abdominal malignancies.

Aims—Because endocannabinoids regulate intestinal inflammation, we investigated the effect of the cannabimimetic, palmitoylethanolamide (PEA), in a mast competent (+/+) and mast cell deficient (*Ws/Ws*) rat model.

Methods—Rats underwent localized, fractionated intestinal irradiation and received daily injections with vehicle or PEA from 1 day before until 2 weeks after radiation. Intestinal injury was assessed non-invasively by luminol bioluminescence, and, at 2 weeks, by histology, morphometry, and immunohistochemical analysis, gene expression analysis, and pathway analysis.

Results—Compared to +/+ rats, *Ws/Ws* rats sustained more intestinal structural injury ($p=0.01$), mucosal damage ($p=0.02$), neutrophil infiltration ($p=0.0003$), and collagen deposition ($p=0.004$). PEA reduced structural radiation injury ($p=0.02$), intestinal wall thickness ($p=0.03$), collagen deposition ($p=0.03$), and intestinal inflammation ($p=0.02$) in *Ws/Ws* rats, but not in +/+ rats. PEA inhibited mast cell-derived cellular immune response and anti-inflammatory IL-6 and IL-10 signaling, and activated the prothrombin pathway in +/+ rats. In contrast, while PEA suppressed

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non-mast cell derived immune responses, it increased anti-inflammatory IL-10 and IL-6 signaling and decreased activation of the prothrombin pathway in *Ws/Ws* rats.

Conclusions—These data demonstrate that the absence of mast cells exacerbate radiation enteropathy by mechanisms that likely involve the coagulation system, anti-inflammatory cytokine signaling, and the innate immune system; and that these mechanisms are regulated by PEA in a mast cell-dependent manner. The endocannabinoid system should be explored as target for mitigating intestinal radiation injury.

Keywords

Endocannabinoid; palmitoylethanolamide; mast cells; intestine; radiation

INTRODUCTION

Intestinal radiation toxicity remains an important dose-limiting factor during abdominal and pelvic radiotherapy. Despite the fact that technological advances have reduced intestinal exposure and clinical bowel toxicity, intestinal radiation toxicity results in treatment delays and causes substantial patient morbidity. Many different drugs and biological response modifiers have been tested in attempts to reduce radiation-induced bowel toxicity (radiation enteropathy), but thus far, few attempts have met with success [1].

Mast cells and neuroimmune interactions play critical roles in the pathogenesis of intestinal radiation injury [2,3]. Recently, members of the endocannabinoid (eCB) system have attracted attention as important regulators of intestinal inflammation and as mediators of interactions between the nervous system and immune system in the gut and other organs. The eCB system has yet to be studied in the context of radiation enteropathy, however, and no mechanistic information exists about the interactions between the eCB system and mast cells.

The eCB system is an endogenous lipid signaling system, consisting of cannabinoid receptors (CB1 and CB2), endogenous ligands for these receptors (endocannabinoids), and enzymes responsible for endocannabinoid biosynthesis and inactivation [4]. CB1 receptors are expressed predominantly in the central and peripheral nervous systems, where they have been implicated in presynaptic modulation of neurotransmitter release. CB2 receptors are found in highest abundance in immune cells and are involved in modulation of immune function and inflammation. In recent years, an emerging potential for eCB system to modulate gastrointestinal inflammation, motility, visceral sensitivity and pain has gained momentum [5,6].

N-palmitoylethanolamide (PEA) is an important member of the autacoid local injury antagonism amide (ALIAmide) group of endogenously produced anti-inflammatory compounds [7]. More specifically, PEA is an endogenous fatty acid amide analogue of the endocannabinoid anandamide that is synthesized “on-demand” during a variety of inflammatory disease states and produces marked protective properties [8]. The mechanism underlying the anti-inflammatory properties of PEA is complex. For example, although PEA exhibits poor affinity for CB1 and CB2 receptors [9,10], its effects appear to involve direct

or indirect activation of CB1 and CB2 receptors via an “*entourage effect*” resulting from elevated levels of anandamide produced by inhibition of the endocannabinoid degrading enzyme fatty acid amide hydrolase (FAAH) [11]. Furthermore, PEA may reduce inflammation by directly activating peroxisome proliferator activated-receptor (PPAR) alpha [10], transient receptor potential vanilloid type-1 (TRPV1) channels [8,12], and by interacting with the G protein-coupled receptor 55 (GRP55) [13]. Important for this study, a prominent property of PEA is its ability to downregulate mast cell activation. Specifically, PEA reduces mast cell infiltration [14], inhibits mast cell degranulation [15], and suppresses the expression and release of mast cell mediators, including histamine, prostaglandin D(2), tumor necrosis factor(TNF)- α , chymase, tryptase, nerve growth factor, and serotonin [14,16]. Correlative data suggests that PEA is effective in several mast-cell mediated experimental models of inflammation [8], and PEA has been suggested as a novel pharmacological approach to treat mast cell regulated disorders [8,17].

The present study was performed to determine whether exogenously administered PEA confers protection against radiation-induced intestinal injury and to ascertain if mast cells contribute to the mechanism of action of PEA in this model. Our results demonstrate that PEA attenuates intestinal radiation injury in mast cell deficient (*Ws/Ws*) rats, but not in mast cell competent (+/+) rats, and that PEA differentially regulates pathways associated with the immune system, anti-inflammatory cytokines, and coagulation system in the two animal strains. These results primarily serve to explain the mechanisms of side effects in patients undergoing radiation therapy of cancer, but may also have implications in radiological or nuclear emergency scenarios.

METHODS AND MATERIALS

Experimental Design

Male mast cell-deficient (*Ws/Ws*) rats and mast cell-competent (+/+) littermate controls, 10–12 weeks of age with average body weight of 150–175g, were purchased from Japan SLC (Hamamatsu, Japan). *Ws/Ws* animals originate from a rat of the BN/fMai strain with a spontaneous 12-base deletion in one locus of the *c-kit* gene. The mutant rat is crossed with normal (+/+) rats of the Donryu strain, in order to generate homozygous *Ws/Ws* rats. Due to the *c-kit* mutation, homozygous *Ws/Ws* rats lack functional melanocytes and interstitial cells of Cajal in the intestine, and are completely devoid of both mucosal and connective mast cells [18,19].

The animals were housed in conventional cages with free access to tap drinking water and standard rat chow (TD8640, Harlan Teklad, Madison, WI). A pathogen-free environment with controlled humidity, temperature, and 12-to-12 light-dark cycle was maintained. All experimental protocols used in this study were approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee.

After two weeks acclimatization, the rat surgical model for localized small bowel irradiation was created [20]. Briefly, rats underwent bilateral orchiectomy, and a loop of distal ileum was sutured to the inside of the left part of the empty scrotum. The model creates a “scrotal hernia” that contains a 4-cm loop of small intestine that can be irradiated locally without

additional surgery. The intestine remains technically within the abdominal cavity, and the surgical procedure does not cause appreciable long-term structural, functional, cellular or molecular alterations. The model minimizes manipulation during irradiation and produces radiation-induced changes similar to those seen clinically. This model has been extensively used and validated in our laboratory.

After 3 weeks postoperative recovery, rats were anesthetized with isoflurane, and the transposed bowel segment within the “scrotal hernia” was exposed to once daily 3.8 Gy fractionated irradiation on 9 consecutive days, using a Seifert Isovolt 320 X-ray machine (Seifert X-Ray Corporation, Fairview Village, PA), operated at 250 kVp and 15 mA, with 3 mm of added aluminum filtration. The resulting half-value layer was 0.85 mm Cu, and the dose rate was 4.49 Gy/min. The radiation regimen was based on data from previous experiments and was designed to elicit moderate to severe radiation enteropathy [20].

N-palmitoylethanolamide (PEA) was purchased from Cayman Chemical (cat# 90350, Ann Arbor, Michigan). PEA was dissolved in a vehicle composed of Tween 80 (Sigma-Aldrich, St Louis, MO), polyethylene glycol (Sigma-Aldrich, St Louis, MO) and physiological saline (1:1:18 by volume). The drug vehicle was made daily and control rats received vehicle only, without PEA. The dose level of PEA (10mg/kg) was based on studies done by others [8,14].

To test whether PEA administration attenuates early radiation-induced intestinal injury in mast cell competent rats, 26 +/- rats were randomly assigned to two treatment groups: PEA (10mg/kg/day) or vehicle (1 ml/kg/day). To test whether PEA administration attenuates early radiation-induced intestinal injury in mast cell deficient rats, 22 *Ws/Ws* rats were randomly assigned to two groups: PEA (10mg/kg/day) or vehicle (1 ml/kg/day). The dose of PEA used in this study was selected based on the previously shown ability of this dose to significantly inhibit mast cell degranulation, infiltration and activation [14,21], as well as to reduce intestinal motility, inflammation and injury in rodents [22,23]. Both vehicle and PEA were administered intraperitoneally (i.p.), beginning 1 day before the start of radiation, during irradiation (9 days), and for 14 days thereafter. All animals were euthanized 2 weeks after completion of their radiation regimen in accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals.

Assessment of the Intestinal Radiation Response

After euthanasia, specimens of irradiated and unirradiated intestine were procured and fixed in Methanol-Carnoy's solution for histological, morphometric, and immunohistochemical studies. The observation time used in this study (2 weeks) is representative of acute (early) radiation enteropathy in our model system.

Quantitative histopathology and morphometry

Radiation injury score—The overall severity of structural radiation injury was assessed using the radiation injury score (RIS) system. The RIS is a composite histopathological scoring system that provides a global measure of the severity of structural radiation injury. It has been extensively used and validated in our laboratory [24]. Briefly, we assessed and graded (from 0–3) seven histopathologic parameters of radiation injury (mucosal

ulcerations, epithelial atypia, subserosal thickening, vascular sclerosis, intestinal wall fibrosis, ileitis cystica profunda, and lymph congestion). The sum of scores for the individual alterations constitutes the RIS. All specimens were evaluated in a blinded fashion by two separate researchers.

Mucosal surface area—A decrease in the surface area of the intestinal mucosa is a sensitive parameter of small bowel radiation injury [24]. Mucosal surface area was measured in vertical sections using a stereologic projection/cycloid method as described by Baddeley [25] and adapted by us for use in our model system. This technique does not require assumptions about the shape or orientation distribution of the specimens and thus circumvents problems associated with most other procedures for surface area measurement.

Thickness of the intestinal wall and subserosa—Intestinal wall thickening is a measure of both reactive intestinal wall fibrosis and intestinal smooth muscle cell hyperplasia. In contrast, subserosal thickening reflects mainly reactive fibrosis. Intestinal wall thickness and subserosal thickness were measured with computer-assisted image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). All measurements were made using a 10X lens. A total of 5 areas, 500 μm apart, were selected for measurement, with 3 measurements taken per area. The average of all 5 areas was used as a single value for statistical calculations.

Quantitative histochemistry and immunohistochemistry and image analysis

Computer-assisted image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD) for histochemistry and immunohistochemistry was used to assess the following established indicators of intestinal radiation injury: 1) proliferation rate of intestinal smooth muscle cells; 2) deposition of collagen in the intestinal wall; and 3) expression of extracellular matrix-associated transforming growth factor- β (TGF- β) as described in detail previously.

Immunohistochemical staining was performed with a standard avidin-biotin complex (ABC) technique, diaminobenzidine (DAB) chromogen, and hematoxylin counterstaining.

Appropriate positive (e.g., psoriatic skin for TGF- β) and negative controls (omission of primary antibody and substitution of primary antibody with non-immune rabbit IgG) were included. The primary antibodies, incubation times, dilutions, and sources were as follows: monoclonal antibody against proliferating cell nuclear antigen (PCNA) (NA03, 2 hrs, 1:100, Calbiochem, Cambridge, MA) and polyclonal antibody against TGF- β (AB-100-NA, 2 hrs, 1:300 dilution, R&D, Minneapolis, MN).

Smooth muscle cell proliferation—In the intestine, collagen is mainly produced by intestinal smooth muscle cells, rather than by fibroblasts [26]. Intestinal smooth muscle cell proliferation rate is very low at baseline, but increases steeply after irradiation (1). Intestinal smooth muscle cell proliferation was assessed in *muscularis propria*. The numbers of total smooth muscle cells and PCNA-positive smooth muscle cells were determined in 20 fields at 40X magnification using color thresholding and normalizing PCNA-positive smooth muscle cells per thousand smooth muscle cells.

Expression of TGF- β —TGF- β is overexpressed in many fibrotic conditions, including radiation fibrosis and is mechanistically involved in radiation enteropathy [27]. Areas relatively positive for TGF- β were determined in 20 fields (40X) according to procedures described by Raviv *et al.* [28], adapted for use in our model system.

Collagen deposition—After irradiation of normal tissues, collagen accumulation is primarily a late endpoint. In the irradiated intestine, however, accumulation of collagen occurs as reactive changes after only 2 weeks. Masson's trichrome method was used for collagen staining. The percentages of areas (relative to the total intestinal wall area) positive for collagen were determined in 20 fields (40X magnification), according to procedures established in our laboratory.

Intestinal mucosal mast cells—Four μ m sections were deparaffinized, rehydrated in graded ethanol, rinsed in distilled water stained in 0.5% of Toluidine blue O (cat# 641, Allied Chemical Corporation, New York, NY) for 7 days, as described. Intestinal mucosal mast cells (MMC) were counted using computer-assisted image analysis. The numbers of MMC per each slide were determined in 10 fields at 40X magnification under a light microscope.

Bioluminescence imaging of myeloperoxidase activity *in vivo*

Neutrophils are an indicator of acute inflammation in irradiated intestine. Myeloperoxidase (MPO) is a well-documented inflammation marker. MPO activity in leukocytes correlates directly with neutrophil number ($r = 0.99$), and MPO activity in tissue extracts correlates directly with neutrophil infiltration when assessed histologically ($r = 0.94$) [29]. Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) is a redox-sensitive compound that emits blue luminescence ($\lambda_{\text{max}} = 425 \text{ nm}$) when exposed to activated MPO. Luminol bioluminescence is uniquely specific for MPO [30]. Briefly, 12 days after irradiation, rats were anaesthetized (isoflurane inhalation) and luminol was administered by i.p. injection (200 mg/kg body weight). Five minutes after luminol injection, the irradiated area of each animal was imaged for MPO activity using the IVIS 200 bioluminescence imaging system (Xenogen). Images were quantified with Living Image Software (Caliper life Sciences, Hopkinton, MA). Areas of luminescence were identified as regions of interest (ROIs) and quantified as photons emitted.

Enzyme-linked Immunosorbent assay (ELISA) for CB1 and CB2 Receptor Detection

CB1 and CB2 were quantified with sandwich ELISA assay according to the manufacturer's instructions (cat# ABIN433277 for CB1 and cat# ABIN434138 for CB2, Antibodies Online Inc, Atlanta, GA). Briefly, frozen small intestinal samples were homogenized in ice-cold PBS containing protease inhibitors and subsequently sonicated using a cell disrupter. Supernatants (100 μ g of total protein) were used for the ELISA assay.

The samples and standards were added to microtiter plate wells precoated with monoclonal antibodies to CB1 and CB2. After 2 hours incubation with primary antibody, a biotin-conjugated polyclonal antibody to CB1 or CB2 (1:100) was added for one hour (CB1 and CB2). Following washing to remove unbound secondary antibody, a solution of avidin

conjugated to Horseradish Peroxidase (HRP) was added for 30 minutes (CB1 and CB2). A TMB substrate solution was added after extensive washing of wells. A change in color (measured spectrophotometrically at a wavelength of 450nm) indicated the presence of CB1 or CB2 receptor–biotin conjugated antibody-HRP conjugated avidin complexes.

Gene Expression Array and IPA Pathway Analysis

Total RNA was extracted from unirradiated and irradiated intestines, using RNeasy Microarray Tissue Mini Kit RNA (Qiagen Sciences, MD). RNA samples were purified to remove contaminating DNA by incubation with Ambion TURBO™ Dnase (Ambion, TX) and their integrity was evaluated by a Agilent Bioanalyzer 2100 (Agilent Technologies, CA). Only samples with RIN number >9.9 and 28S/18S >2.0 were used. Transcriptional profiles were analyzed using Genespring GX 11.0 software (Agilent Technologies, CA). Raw data were log2 transformed and then normalized to the 75th percentile of all values on an individual chip. A list of genes with 2.0-fold change was generated and tested by the Benjamini-Hochberg Multiple Testing Correction procedure. Significant genes were selected by a cut-off of $p < 0.05$ and fold change ≥ 2.0 . Gene pathway analysis was performed by use of Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems Inc., CA). Interrogation of the relationship of PEA and mast cell on the mediation of canonical pathways during acute radiation injury, comparison analyses were performed between $+/+$ and Ws/Ws rats, and between $+/+$ and Ws/Ws rats treated with PEA or vehicle. Investigated pathways included those that regulate metabolism, cellular cycle control, cellular injury and DNA damage repair, immune response, and cytokine production. IPA Downstream Effect Analysis was performed to evaluate the global impact of PEA on radiation-induced biological process regulation in $+/+$ and Ws/Ws rats. The p-value associated with functions, pathways, or lists in IPA was calculated using the right-tailed Fisher Exact Test. p-values less than 0.05 indicate a statistically significant, non-random association.

Statistical Analyses

Sample size estimates for these experiments were generated with PASS 11 (NCSS, Kaysville, UT) using data from previous experiments performed in our laboratory as “seeds”. Subsequent data analysis was performed with StatXact 8 (Cytel, Cambridge, MA) or with NCSS 2007 (NCSS) when no non-parametric alternative was available in StatXact. Gene expression data were analyzed with built-in statistical routines in the IPA software (Ingenuity).

RESULTS

Radiation or PEA administration did not result in significant body weight differences between $+/+$ rats and Ws/Ws rats ($p > 0.05$, respectively). In vehicle-treated $+/+$ rats an intestinal perforation was observed in one animal, while no perforations were observed in PEA-treated animals. In vehicle-treated Ws/Ws rats, one animal died and three rats were observed to have perforations. No death or perforations occurred in PEA-treated Ws/Ws rats.

In unirradiated intestine specimens, an abundance of mast cells was observed in the intestinal mucosa obtained from +/+ rats and PEA administration did not alter the number of mucosal mast cells ($p>0.05$). No mast cells were observed in intestinal mucosa of vehicle- or PEA-treated *Ws/Ws* rats.

Intestinal CB1 and CB2 Receptor Expression in +/+ and *Ws/Ws* Rats

Unirradiated intestines from +/+ rats and *Ws/Ws* rats expressed measureable levels of CB1 and CB2 receptor protein, however, the level of CB1 receptors was much lower than that of CB2 receptors in both strains (Figure 1A), which were consistent with RNA expression (data not shown). The levels of CB1 and CB2 receptors in unirradiated intestine from +/+ rats were higher than that observed in *Ws/Ws* rats ($p<0.01$ for CB1 and $p=0.05$ for CB2) (Figure 1A). Radiation did not impact the levels of CB1 and CB2 receptor in either +/+ rats or *Ws/Ws* rats ($p>0.05$, respectively) (Figure 1B and 1C).

Effects of Radiation in +/+ Rats and *Ws/Ws* Rats

Compared to +/+ rats, in response to irradiation, *Ws/Ws* rats demonstrated increased overall intestinal structural injury (RIS, $p=0.01$), reduced intestinal surface area ($p=0.02$), enhanced influx of neutrophils into irradiated area ($p=0.0003$) and exacerbated collagen deposition ($p=0.004$) (Figure 2). These findings are consistent with those reported by our laboratory previously (1).

Effects of PEA in +/+ Rats

Radiation-induced histopathologic changes in +/+ rats were similar to those observed in prior studies published from our laboratory. Early alterations (2 weeks) post radiation consisted primarily of mucosal injury (as measured by mucosal surface area), reactive intestinal wall thickening (as measured by intestinal wall thickening), inflammatory cell infiltration (as total MPO influx measured by bioluminescence), increased smooth muscle cell proliferation, and excessive deposition of collagens and TGF- β in the intestinal wall ($p<0.001$ for all parameters, data not shown).

PEA administration did not alter overall radiation-induced structural injury, intestinal surface area, intestinal or serosal thickness, collagen deposition, intestinal smooth muscle proliferation, and TGF- β immunoreactivity (Figure 3). The influence of PEA administration on neutrophil influx into irradiated intestine also did not reach statistical significance ($p=0.06$) (Figure 4).

Effects of PEA in *Ws/Ws* Rats

In contrast to effects observed in +/+ rats, in *Ws/Ws* rats PEA administration significantly reduced overall radiation structural injury ($p=0.02$), intestinal wall thickness ($p=0.03$), collagen deposition ($p=0.03$) and neutrophil influx into irradiated intestinal areas ($p=0.02$) (Figure 3 and Figure 4). A trend towards improvement of intestinal surface area was also apparent, but did not reach statistical significance ($p=0.06$). PEA administration had no effect on radiation-induced changes in intestinal serosal thickness, intestinal smooth muscle proliferation and TGF- β immunoreactivity (Figure 3).

Gene Expression Array Analysis

Gene expression profile analysis—In vehicle treated *+/+* rats, radiation exposure induced a greater than 2-fold change in 985 genes (555-up, 430-down). PEA administration reduced the number of genes altered to 609 (418-up, 191-down). In the vehicle treated *Ws/Ws* rats, radiation induced a greater than 2-fold change in 983 genes (482-up, 501-down) and PEA administration only slightly decreased the number of altered genes to 996 (563-up, 433-down).

IPA Downstream Effect Analysis—IPA Downstream Effect Analysis to evaluate the global impact of PEA on radiation-induced alterations of biological processes in *+/+* and *Ws/Ws* rats. From the heatmap generated (Supplementary Figure 1), it is observed that radiation injury induced a broad up-regulation of genes involved in hematological system development and function, cellular movement, immune cell trafficking, cell-to-cell signaling and interaction and inflammatory response in intestines obtained from both *+/+* and *Ws/Ws* rats. Up-regulation of these genes was only slightly attenuated in *+/+* rats, but significantly suppressed in *Ws/Ws* rats. In addition, radiation injury induced a slight down-regulation of genes involved in small molecular chemistry, lipid metabolism and molecular transport in intestines of *+/+* rats, but dramatically decreased the abundance of genes associated with these functions in *Ws/Ws* rats.

IPA Canonical Pathway Analysis—To study the impact of PEA on canonical pathway activation during radiation injury, a comparison pathway analysis was performed on the expressional profiles in intestines obtained from *+/+* and *Ws/Ws* rats, as well as from vehicle and PEA-treated rats in both strains. Pathways that mediate the immune response, cytokine production, cellular stress and injury were selected for analysis. Compared to *+/+* rats, a stronger activation of cellular stress and injury pathways, including the coagulation system and intrinsic and extrinsic prothrombin activation pathways was observed in *Ws/Ws* rats (Supplementary Figure 2A). The up-regulated coagulation system and intrinsic and extrinsic prothrombin activation pathways were attenuated by PEA administration in *Ws/Ws*, whereas the up-regulated intrinsic and extrinsic prothrombin activation pathways were exacerbated by PEA in *+/+* rats (Supplementary Figure 2B & C).

Pathway analysis also revealed that, compared to *+/+* rats, the up-regulated pathways that control cellular immune responses (Supplementary Figure 3A) and cytokine signaling (Supplementary Figure 4A) were significantly attenuated in *Ws/Ws* rats. PEA administration reduced up-regulation of these pathways in both rat strains (Supplementary Figure 3B & C, Supplementary Figure 4B & C). However, in *Ws/Ws* rats PEA increased IL-10 and IL-6 signaling (Supplementary Figure 4C).

DISCUSSION

Mast cells play a critical role in immune regulation and inflammation [31,32]. Although best known for their function in allergy and anaphylaxis, mast cells exert a significant protective function [33]. We have previously shown that mast cells protect against early intestinal radiation-induced injury [2,34]. Because we hypothesized that the eCB system would influence radiation enteropathy development and PEA is a known mast cell modulator [8],

we surmised that exogenously administered PEA regulate radiation enteropathy in a mast cell dependent manner. The present study investigated the effects of PEA $+/+$ and Ws/Ws rats. The major findings were that; 1) mast cells protect against intestinal radiation-induced injury, consistent with previous results, and 2) PEA attenuates intestinal radiation injury in Ws/Ws rats, but not in $+/+$ rats. Hence, the effect of PEA to ameliorate radiation-induced intestinal injury is opposed by exacerbating effects mediated via mast cells. The balance produced by these opposing effects explains why PEA attenuates intestinal injury in Ws/Ws rats, but not in $+/+$ rats.

The mechanism(s) responsible for the anti-inflammatory actions of PEA are complex (see Introduction) [10]. Therefore, the protective effects of PEA against radiation-induced intestinal injury reported here likely result from the action of PEA at multiple targets. For example, PEA has been shown to indirectly and/or directly activate CB1 [35,36] and CB2 receptors [8,37] via an “*entourage effect*” producing elevated levels of the endocannabinoid anandamide [11]. Activation of CB1 receptors inhibits development of colitis induced by mustard oil and dextran sulfate sodium [38], and CB1-deficient mice show elevated levels of intestinal CB1 receptors and enhanced inflammation in response to chemically-induced colitis [39]. Furthermore, CB2 receptor activation on macrophages, neutrophils and lymphocytes may produce general anti-inflammatory and immunosuppressive effects [40]. For example, studies have shown that CB2 receptor activation augments the production of the anti-inflammatory cytokines IL-4 and IL-10 and reduces release of pro-inflammatory cytokines, chemokines and molecules, including IL12p40, tumor necrosis factor- α , IL-1 β , and interferon- γ [41–43] and reactive oxygen species [44]. Finally, *in vivo* studies demonstrate that PEA suppresses the expression of inflammatory mediators such as TNF- α , IL-1 β , cyclooxygenase-2, and inducible nitric-oxide synthase [45,46]; increases phagocytosis of bacteria by macrophages [47]; inhibits neutrophil infiltration; and reduces enterocyte apoptosis [23]. PEA regulates local and systemic inflammation, including intestinal injury [48]. Since our results demonstrate that both cannabinoid receptors are present in the intestine and their levels are not altered by radiation-injury, the protective effects of PEA reported here might result from reduction in inflammation due to direct and/or indirect activation of intestinal CB1 and CB2 receptors.

The protective effects of PEA observed in this study might also be due to direct activation of PPAR- α , which would be predicted to attenuate inflammation [10]. PPAR- α modulates inflammation by directly interacting with pro-inflammatory transcription factors NF- κ B and activator protein 1 [48]. PPARs are expressed in rat intestine at various levels [49] and PPAR- α ligands play a protective role in experimental inflammatory bowel diseases [50].

Finally, PEA could protect against radiation-induced injury through modulation of TRPV1 function. Emerging evidence demonstrates protective functions for this unique receptor *in vivo*, such as for LPS-induced sepsis [51], ischemia/reperfusion injury [52], and experimental colitis [53]. Our laboratory has shown that sensory nerve ablation (to eliminate TRPV1 receptors) exacerbates intestinal radiation injury [34]. Thus, PEA might exert beneficial effects on radiation-induced intestinal injury by activating TRPV1 receptors.

The possibility that the response to PEA in *Ws/Ws* rats may be due in part to the fact that these rats also exhibit a loss of interstitial cells of Cajal (ICC) should also be considered. There is little information regarding how these cells affect the radiation response. Further studies should be performed, for example, with selective ablation of ICCs, to investigate this issue.

Taking all postulated mechanisms into account, the effects resulting from PEA-induced mast cell inhibition in *+/+* rats might be counterbalanced by anti-inflammatory or protective effects produced by mediators derived from other immune cells. In other words, while PEA likely exerts a protective effect on most immune cells, we surmise that this is counterbalanced by its inhibitory effects on mast cells in the intestinal mucosa.

Indeed, IPA Canonical Pathway analysis revealed that radiation up-regulated cellular immune response pathways in *+/+* rats, whereas all up-regulated pathways were suppressed in *Ws/Ws* rats. These pathways are central to mounting an efficacious immune response to bacterial infections and inflammatory stimuli [53–57]. Suppression of these pathways in *Ws/Ws* rats suggests that mast cells contribute in a major way to the physiological immune response and may explain why injury is more severe in *Ws/Ws* rats.

Comparison of gene expression also demonstrated that in *Ws/Ws* rats, PEA strongly attenuated up-regulation of multiple radiation-induced cellular immune response pathways, while increasing IL-10 and IL-6 signaling. Interestingly, these observations were opposite to the effects produced by PEA in *+/+* rats. IL-10 and IL-6 are important cytokines in inflammation and immune regulation [58,59].

In addition to modulation of the immune response and cytokine production, radiation-induced cellular stress and injury pathways were highly affected by PEA in both *+/+* and *Ws/Ws* rats. Radiation exposure induced an up-regulation of the coagulation system, intrinsic and extrinsic prothrombin pathways in *+/+* rats, and mast cell deficiency exacerbated up-regulation of these pathways. These observations are consistent with a role of mast cells as antithrombotic cells [60]. Interestingly, in *+/+* rats, PEA exacerbated up-regulation of prothrombin activation pathways, whereas it significantly attenuated up-regulation of the coagulation system in *Ws/Ws* rats. PEA-treated *+/+* rats and vehicle-treated *Ws/Ws* rats showed a similar expression pattern in the prothrombin activation pathways, indicating that PEA inhibits mast cell-regulated prothrombin activation pathways in *+/+* rats. Endothelial cells express CB2 receptors and activation of CB2 receptors appears to limit endothelial inflammatory responses, resulting in the restoration of endothelial function [61]. Therefore, a beneficial effect of PEA on endothelial protection in *+/+* rats might be counteracted by a detrimental effect due to the inhibition of mast cell-derived anticoagulation. This may help to explain why PEA-treatment failed to produce a net beneficial effect on intestinal radiation injury in *+/+* rats. Conversely, since mast cells are absent in *Ws/Ws* rats, the beneficial effects of PEA on endothelial protection might dominate.

IPA Downstream Effect Analysis demonstrated a global effect of PEA as reflected by greater than 2-fold change in many genes associated with radiation-induced biological

functions. In +/- rats, PEA slightly suppressed radiation-induced effects on hematological system development and function, cellular movement, immune cell trafficking, cell-cell interaction and inflammatory responses. In marked contrast, these functions were significantly suppressed in *Ws/Ws* rats. Up-regulation of these functions was almost entirely suppressed by PEA in *Ws/Ws* rats, suggesting the importance of non-mast cell mediators in the regulation of these functions. Furthermore, suppression of these functions may result in a net effect to reduce inflammatory cell infiltration, leading to reduced intestinal radiation injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Protein levels of CB1 and CB2 receptors in vehicle-treated unirradiated and irradiated intestines from +/+ rats (N=9) and *Ws/Ws* (N=8) rats. Irradiated and unirradiated intestines from vehicle-treated +/+ and *Ws/Ws* rats were procured 2 weeks after the completion of irradiation. Protein levels were measured ELISA. A. Comparison of CB1 and CB2 levels in unirradiated intestines from +/+ and *Ws/Ws* rats; B. Levels of CB1 receptors in unirradiated and irradiated intestines from +/+ and *Ws/Ws* rats; C. Levels of CB2 receptors in unirradiated and irradiated intestines.

Figure 2.

Radiation effects on intestines in +/+ rats and *Ws/Ws* rats (mean, standard error of mean). *p* values refer to difference between +/+ rats and *Ws/Ws* rats. Irradiated intestines from vehicle-treated +/+ and *Ws/Ws* rats were procured 2 weeks after the completion of irradiation. A. Radiation injury score (a composite histopathologic score); B. Mucosal surface area; C. Intestinal wall thickness; D. MPO activity by bioluminescence; E. Collagen deposition.

Figure 3.

Effect of PEA (10 mg/kg/d) on radiation-induced intestinal injury in +/+ rats and *Ws/Ws* rats (mean, standard error of mean). Irradiated intestines from vehicle-treated and PEA-treated +/+ and *Ws/Ws* rats were procured 2 weeks after the completion of irradiation. A. Radiation injury score; B. Intestinal wall thickness; C. Mucosal surface area; D. Collagen deposition. Representative histological images of intestines from *Ws/Ws* rats treated with vehicle or PEA are shown.

Figure 4. Bioluminescence image of *in vivo* MPO activity on vehicle- and PEA-treated (10 mg/kg/d) +/+ rats and Ws/Ws rats (mean, standard error of mean). Quantitative total MPO flux 12 days after radiation exposure.