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The PPAR δ agonist, GW0742, inhibits neuroinflammation, but does not restore neurogenesis or prevent early delayed hippocampal-dependent cognitive impairment after whole-brain irradiation

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Abstract

Brain tumor patients often develop cognitive impairment months to years after partial or fractionated whole-brain irradiation (fWBI). Studies suggest that neuroinflammation and decreased hippocampal neurogenesis contribute to the pathogenesis of radiation-induced brain injury. In this study, we determine if the peroxisomal proliferator-activated receptor (PPAR) δ agonist, GW0742, can prevent radiation-induced brain injury in C57Bl/6 wild-type (WT) and PPAR δ knockout (KO) mice. Dietary GW0742 prevented the acute increase in IL-1 β mRNA and ERK phosphorylation measured at 3 h after a single 10 Gy dose of WBI; it also prevented the increase in the number of activated hippocampal microglia 1 week after WBI. In contrast, dietary GW074 failed to prevent the radiation-induced decrease in hippocampal neurogenesis determined 2 months after WBI in WT mice, or mitigate their hippocampal-dependent spatial memory impairment measured 3 months after WBI using the Barnes maze task. PPAR δ KO mice exhibited defects including decreased numbers of astrocytes in the dentate gyrus/hilus of the hippocampus and a failure to exhibit a radiation-induced increase in activated hippocampal microglia. Interestingly, the number of astrocytes in the dentate gyrus/hilus was reduced in WT mice, but not

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in PPAR δ KO mice 2 months after WBI. These results demonstrate that, although dietary GW0742 prevents the increase in inflammatory markers and hippocampal microglial activation in WT mice after WBI, it does not restore hippocampal neurogenesis or prevent early delayed hippocampal-dependent cognitive impairment after WBI. Thus, the exact relationship between radiation-induced neuroinflammation, neurogenesis, and cognitive impairment remains elusive.

Keywords

(PPAR) δ agonists; C57Bl/6 wild-type mice; PPAR δ KO mice; whole-brain irradiation; cognitive impairment; neuroinflammation; neurogenesis

Introduction

Partial or fractionated whole-brain irradiation (fWBI) often is required to treat both primary and metastatic brain cancer [1]. However, radiation-induced normal brain injury, including progressive cognitive impairment, can affect the quality of life (QOL) of patients who receive fWBI [2-4]. This diminished QOL has become an important concern for long-term survivors of brain irradiation and is recognized as one of the most important measures of brain tumor therapy outcomes in clinical trials [5]. Radiation-induced cognitive impairments often present as deficits in executive function, spatial learning/memory, verbal memory, attention/concentration, fatigue, and/or mood [6-10]. Although clinical trials have demonstrated that short-term interventions after fWBI can modulate these cognitive impairments, there are no proven long-term treatments for radiation-induced cognitive impairment [11]. Thus, it is important to investigate the mechanism(s) responsible for radiation-induced brain injury, including cognitive impairment, and develop new therapeutic strategies to prevent/ameliorate it [12]

Neuroinflammation is hypothesized to play a key role in the brain's response to radiation. In rodents, this neuroinflammatory response is often characterized by increases in the number of activated hippocampal microglia [13, 14] that modify the brain's microenvironment and have the ability to alter many neuronal functions [15-17]. Rodent studies also show that there is a negative correlation between an increase in hippocampal microglial activation and a decrease in hippocampal neurogenesis after irradiation [15]. Moreover, this radiation-induced decrease in hippocampal neurogenesis has been shown to be associated with defects in hippocampal-dependent learning and memory [18]. Anti-inflammatory drugs have been shown to inhibit radiation-induced increases in microglial activation, and concomitantly, prevent/ameliorate radiation-induced decreases in hippocampal neurogenesis in rodents [15, 19]. Thus, drug therapies that reduce inflammation are likely to be beneficial in preventing/ameliorating radiation-induced brain injury, including cognitive impairment.

The anti-inflammatory and anti-oxidant properties of peroxisomal proliferator-activated receptor (PPAR) δ agonists have been characterized in multiple cell types [20]. Furthermore, administration of PPAR δ agonists have been shown to confer neuroprotection in both acute and chronic diseases of the central nervous system, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, and stroke, predominantly by modulating inflammatory markers associated with these diseases [21-24]. We previously reported that

pre-treating BV-2 murine microglial cells *in vitro* with the PPAR δ agonist, L-165041 prevented radiation-induced increases in inflammatory markers by negative regulation of the NF- κ B and PKC α /MEK/ERK/AP-1 pathways [25]. Unpublished data from our lab also demonstrate that pretreating primary rat astrocytes with L-165041 inhibits radiation-induced increases in IL-6 and MCP-1 mRNA in part by preventing activation of the PKC α /MEK1/2/ERK1/2 pathway.

Based on these *in vitro* results, we hypothesized that dietary administration of the PPAR δ agonist, GW0742, to 12-16 week old male C57Bl/6 mice will prevent/ameliorate a, i] neuroinflammatory response, ii] decrease in hippocampal neurogenesis, and iii] hippocampal-dependent cognitive impairment measured after a single 10 Gy dose of whole-brain irradiation (WBI). Although dietary GW0742 prevented the radiation-induced acute hippocampal inflammatory response, it did not prevent the decrease in hippocampal neurogenesis or the early delayed hippocampal-dependent cognitive impairment after WBI.

Materials and Methods

Animals and irradiation procedures

Adult (12-16 week old) C57Bl/6 wild-type (WT) mice (Jackson Laboratories, Bar Harbor, Maine) and PPAR δ knockout (KO) mice (NIH, Bethesda, Maryland) were housed in a specific pathogen free environment, 5 mice per cage with free access to drinking water and standard mouse chow (Harlan Teklad, Madison, WI), with or without the PPAR δ agonist, GW0742 (GlaxoSmithKline, Research Triangle Park, NC); diets were prepared in our Diet Core Lab at the Primate Center. All animal handling and experiments were performed in strict accordance with the NIH Guide for Care and Use of Laboratory Animals as approved by the Wake Forest School of Medicine Institutional Animal Care and Use Committee. Mice were randomized to 4 experimental groups: 1) sham-irradiation and control diet, 2) sham-irradiation and 100 ppm of GW0742 in the diet, 3) WBI and control diet, and 4) WBI and dietary GW0742. The composition of the control diet has been described previously [19]. Mice were placed on their respective diets 14 days prior to WBI and maintained on their diets until euthanized at 3 h, 1 week, 2 months or 6 months after WBI. Mice were anesthetized using a ketamine/xylazine mixture (90/10 mg/kg body weight [BW]) injected i.p. prior to irradiation. WBI was performed, as previously described, using a 7,214 Ci self-shielded ^{137}Cs irradiator with lead and Cerrobend shielding devices [19]. Irradiated mice received a single dose of 10 Gy at a dose rate of ~ 4 Gy/min with 5 Gy delivered to each side of the head. Sham-irradiated mice were anesthetized, but were not irradiated.

Bromodeoxyuridine (BrdU) injection

One month after WBI, mice received an i.p. injection of 50 mg/kg of BrdU every day for 7 days. Two months after WBI (3 weeks after the last BrdU injection), the mice were euthanized, and the tissues processed as described below.

Tissue processing

Mice were euthanized by an i.p. injection of a ketamine/xylazine mixture (200/10 mg/kg BW) followed by cervical dislocation. For CD68 and NeuN/BrdU staining, the brains were

excised, the right hemisphere of the brain was fixed with 4% paraformaldehyde, cryoprotected in 10%, 20%, and 30% sucrose, and then frozen in tissue embedding medium. Coronal sections containing the hippocampus (40- μ m thickness) were sectioned using a cryostat, collected in anti-freeze solution (1:1:2 ethylene glycol, glycerol and 0.1 M phosphate-buffered saline), and stored at -20°C . The left hemisphere was snap frozen in liquid nitrogen, to be used for harvesting RNA and protein.

Immunohistochemistry and immunofluorescence

A 1-in-6 (CD68⁺ and GFAP⁺ cells) or a 1-in-12 (NeuN⁺/BrdU⁺ cells) series of sections from the anterior-to-posterior of the dentate gyrus (DG) were chosen based on systematic random sampling. Tissue sections were washed in Tris-buffered saline (1X TBS; pH 7.4). For immunohistochemical staining, sections were treated with 1% H_2O_2 in 1X TBS to block endogenous peroxidase activity and incubated overnight at 4°C with either a rat α -CD68 antibody (labels activated microglia; 1:100; AbD Serotec, Raleigh, NC), or a rabbit α -GFAP antibody (labels astrocytes; 1:7000; Dako, Carpinteria, CA). The primary antibodies were detected using a biotinylated secondary antibody (1:200, Vectorlabs, Burlingame, CA) and visualized using peroxidase-conjugated avidin-biotin complex (ABC Elite kit, Vectorlabs) with nickel-enhanced DAB substrate (Vectorlabs). For immunofluorescence staining, sections were treated with 2 M HCl at 37°C to denature the DNA, washed in 1X TBS pH 8.5 to neutralize the acid, incubated for 2 h in blocking solution (10% normal serum, 0.3% Triton X-100 in 1X TBS), and finally incubated overnight at 4°C with rat α -BrdU (1:200; AbD Serotec, Raleigh, NC) and mouse α -NeuN (1:200; Chemicon, Billerica, MA) antibodies. BrdU and NeuN labeling were detected using Cy3 and Alexa-Fluor® 488, respectively (1:200, Jackson ImmunoResearch, West Grove, PA). DAPI (4', 6-diamidino-2-phenylindole, Sigma-Aldrich, St. Louis, MO), a DNA binding fluorescent dye, was used to visualize the DG anatomical landmarks.

Unbiased stereology

Unbiased stereology is considered superior to traditional, single-section estimation techniques because it is not biased by the shape, size, cell orientation, or volumetric variation [26]. This allows an accurate estimate of cell numbers with small sample sizes. In this study, unbiased stereology was used to quantify the number of activated microglia (CD68⁺) and astrocytes (GFAP⁺) in the granule cell layer (GCL) and hilus of the DG. Using a Zeiss Imager D2 microscope with StereoInvestigator software (Microbrightfield, Inc, Colchester, VT), cell counts were performed by investigators that were blinded to the experimental condition of the sample. The counting parameters for CD68⁺ or GFAP⁺ cells in the GCL/hilus were as follows: sampling grid size (100 $\times$ 100 μm), counting frame size (75 $\times$ 75 μm), disector height (15 μm), and guard-zone thickness (2 μm). The coefficient of error (CE) was determined using the Gundersen-Jensen CE estimator to obtain the precision of the stereological counts. The variance introduced by the stereological analysis should not account for more than 50% of the observed group variance, i.e. the ratio between CE^2 and observed variance of the group, CV^2 , should be < 0.5 [27, 28]. All values in the current study were < 0.5 .

The BrdU⁺ and BrdU⁺/NeuN⁺ cells were counted in the GCL and subgranular zone (SGZ) of the hippocampus in stacks of optical sections acquired using a Leica TCS SP2 confocal microscope (Leica Microsystems, Bannockburn, IL). The sum of the BrdU⁺/NeuN⁺ cells in the sampled sections from each mouse was determined, and the average value for each treatment group plotted and analyzed.

RNA isolation and qRT-PCR Syber Green

RNA was harvested using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. DNA contamination was removed by acid-phenol chloroform extraction (pH 4.6, 125:24:1, Ambion Inc., Austin, TX). Real-time PCR amplifications were conducted in a 25 µL reaction volume containing 1 µL cDNA, 12.5 µL SYBR Green PCR Master Mix (Roche, Indianapolis, IN), 0.1 µM upstream and downstream primers, and 10.5 µL of nuclease-free water. Real-time PCR was carried out in an ABI Prism® 7000 at 50°C for 2 min, 95°C for 2 min, and then 45 cycles of 95°C for 15 min, 55°C for 30 sec, and 72°C for 30 sec. The relative expression of IL-1β gene expression was calculated using the comparative C_t method (25); the loading control was β actin.

Immunoblotting

Total cellular protein was harvested using M-PER lysis buffer (Pierce Biotechnology, Inc., Rockford, IL) supplemented with 1 mg/mL aprotinin (Sigma-Aldrich), 1 mg/mL leupeptin (Sigma-Aldrich), 10 mg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄ (Sigma-Aldrich), and 150 mM of NaCl. Lysates were centrifuged at 12,500 rpm for 10 min, and the supernatant collected. Protein concentrations were measured using the Bradford assay (Bio-Rad, Hercules, CA); the absorbance was measured at 595 nm. Proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes for 1.5-3 h at 80 V, blocked in 2.5% BSA in TBST (0.02 M Tris, 0.015 M NaCl, 0.05% Tween 20, pH 7.5), and incubated overnight with p-ERK primary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Membranes were washed, incubated with the appropriate HRP-conjugated secondary antibody, developed using the ECL detection system (GE Healthcare, NJ), and processed using a Kodak Processing System. Films were scanned, and densitometry performed to quantify the signal intensity using Adobe Photoshop Elements 6.0.

Barnes maze task

The Barnes maze task is a sensitive test for detecting hippocampal-dependent spatial learning and memory impairments following irradiation [18, 29]. The Barnes maze consists of a 121.9 cm diameter white Plexiglas circular arena with 18 equidistant holes. These holes are 5.5 cm in diameter, spaced every 20°, and centered 4.5 cm from the outer perimeter. A black Plexiglas escape box (30 × 14 × 6 cm) was positioned beneath one target hole; this position is fixed for each mouse. A unique picture was positioned on each cardinal compass point to provide visual cues. The Barnes maze was brightly illuminated with two 34 W diffuse overhead fluorescent lights. In addition, a white noise generator was used to minimize environmental noise (Target, Minneapolis, MN).

On Day 1, mice were habituated to the Barnes maze and the existence of an escape box. Mice were placed in the center of the Barnes maze under a clear container for 30 sec. After 30 sec, the container was lifted, and the mice allowed to explore the maze for 1 min. If the mice did not find the escape box in 1 min, they were guided to it, and a cover placed over the hole to prevent them from getting out for 1 min. This was repeated 3 times, and the data recorded as the Day 1 trial. On Day 2-5 and 7-11, the mice performed two 3 min trials. A trial was ended when the full body of a mouse entered into the escape box. If a mouse did not locate the escape box within 3 min, it was guided to it and prevented from getting out as on Day 1. On Day 6 and Day 12, mice performed a 1 min probe trial with the escape box removed. The probe trial measures the frequency in the zone where the escape box was previously located, providing an additional measure of spatial learning and memory. The Barnes maze was wiped with 100% ethanol between each trial to prevent a mouse from using olfactory cues to locate the escape box. The behavior of the mice in the Barnes maze was recorded by a video camera using an automated video tracking system and analysis software (Ethovision, Nodulus, Leesburg, VA).

Statistical Analysis

For all immunohistochemical and molecular data, analyses were carried out using SAS software (SAS Inc, Cary, NC). The conditional normality was checked for each data set, and an appropriate transformation performed, if necessary. With the exception of the GFAP+ cell data, a 2-way ANOVA was used to determine if there was a radiation effect, drug effect, and/or any interaction effects. If a significant effect was found, post-hoc pairwise comparisons were performed, controlling for multiple comparisons, using Bonferroni and Tukey's studentized range tests. The GFAP+ cell data was analyzed using an unpaired, equal sample size Student's t test. In all cases, $p < 0.05$ was considered significant.

For all Barnes Maze data, the latency to the tunnel (escape box) over the course of training trials were analyzed using a generalized estimating equation and a two-factor repeated-measures ANOVA (radiation x drug x trial). These analyses were followed by a pairwise comparison, controlling for multiple comparisons, to determine which time points were significantly different from the appropriate comparison group. A 2-way ANOVA was used to analyze the Day 6 and Day 12 probe data to determine if there was a radiation, drug, or interaction effect. In all cases, $p < 0.05$ was considered significant.

Results

GW0742 administration inhibits the WBI-induced increase in IL-1 β and p-ERK

We have reported previously that PPAR δ activation prevents the radiation-induced increase in IL-1 β mRNA and ERK phosphorylation in BV2 microglia cells *in vitro* [25]. Therefore, we examined if dietary GW0742 inhibited these inflammatory markers in the brains of WT mice at 3 h after a single 10 Gy dose of WBI. WT mice exhibited an increase in IL-1 β mRNA at 3 h after WBI (Fig. 1A; radiation effect: $F_{1,8}=11.49$, $p<0.01$; Bonferroni, $p<0.01$). Dietary GW0742 alone had no effect on the IL-1 β mRNA level (Fig. 1A; drug effect: $F_{1,8}=3.92$, $p>0.08$), but prevented the radiation-induced increase in IL-1 β mRNA in WT mice at 3 h after WBI (Fig. 1A; interaction effect: $F_{1,8}=6.92$, $p<0.05$, Bonferroni, $p<0.05$).

WT mice exhibited an increase in ERK phosphorylation at 3 h after WBI (Fig. 1B; radiation effect: $F_{1,8}=6.78$, $p<0.01$, Bonferroni, $p<0.05$). Dietary GW0742 alone had no effect on ERK phosphorylation (Fig. 1B; drug effect: $F_{1,8}=4.28$, $p>0.07$), but prevented the radiation-induced increase in ERK phosphorylation in WT mice at 3 h after WBI (Fig. 1B; interaction effect: $F_{1,8}=8.06$, $p<0.05$, Bonferroni, $p<0.05$). At 1 week and 2 months after WBI, no radiation-induced increase in IL-1 β or ERK phosphorylation was detectable (data not shown). These results demonstrate that the dietary PPAR δ agonist, GW0742, has the ability to prevent an acute inflammatory response after WBI.

Dietary GW0742 prevents the radiation-induced increase in activated microglia in WT mice

Because *in vitro* studies in our lab indicated that PPAR δ agonists can inhibit the radiation-induced inflammatory response in BV2 microglial cells [25], we hypothesized that dietary GW0742 would prevent a radiation-induced increase in activated hippocampal microglia. One week after a single 10 Gy dose of WBI, WT mice exhibited a significant increase in the number of activated microglia (CD68⁺ cells) in the DG/hilus (Fig. 2A; radiation effect: $F_{1,8}=10.31$, $p<0.01$, Bonferroni, $p<0.01$). Dietary GW0742 alone had no effect on the number of activated microglia in the DG/hilus (Fig. 2A; Bonferroni $p>0.05$), but prevented the radiation-induced increase in the number of activated microglia at 1 week after WBI (Fig. 2A; Bonferroni $p<0.001$). Although there was a slight increase in the number of activated hippocampal microglia in WT mice at 2 months after WBI that appeared to be reduced by dietary GW0742, none of the changes were statistically significant (Fig. 2B; radiation effect: $F_{1,8}=0.09$, $p>0.8$; drug effect: $F_{1,8}=5.15$, $p>0.05$; interaction effect: $F_{1,8}=0.90$, $p>0.4$). Thus, dietary GW0742 prevented the radiation-induced increase in activated hippocampal microglia measured 1 week after WBI, but neither a radiation nor a drug effect were detectable at 2 months after WBI.

Activated hippocampal microglia are not increased in irradiated PPAR δ KO mice

It has been reported that PPAR δ agonists' effects can be independent of the receptor [30]. To address this concern, we determined if dietary GW0742 could prevent a radiation-induced increase in the number of activated hippocampal microglia in PPAR δ KO mice. Interestingly, the number of activated microglia in the DG/hilus of KO mice was unaltered by either irradiation or the drug at 1 week (Fig. 2C; radiation effect: $F_{1,8}=0.23$, $p>0.6$, drug effect: $F_{1,8}=0.38$, $p>0.6$) and 2 months (Fig. 2D; radiation effect: $F_{1,8}=0.08$, $p>0.8$; drug effect: $F_{1,8}=0.06$, $p>0.8$) after WBI. The absence of an increase in activated hippocampal microglia in the irradiated KO mice prevented us from determining whether GW0742 modulates radiation-induced hippocampal microglial activation by a PPAR δ -independent mechanism. These findings are quite different from those measured in PPAR α KO mice, where the absence of PPAR α resulted in a sustained increase in the number of activated hippocampal microglia measured at 1 week and 2 months after WBI [19]. These data suggest that the biological effects of PPAR deficiencies are likely to be subtype-dependent.

In addition to displaying a different microglial response than WT mice after WBI, PPAR δ KO mice also exhibited a different astrocytic response. Sham-irradiated WT mice had almost twice as many hippocampal astrocytes (GFAP⁺ cells) as sham-irradiated PPAR δ KO mice (Fig. 3, $p<0.05$). Moreover, WT mice had a significant ($p<0.05$) decrease in the

number of hippocampal astrocytes at 2 months after WBI, but PPAR δ KO mice did not exhibit a further decrease in astrocyte number in response to radiation (Fig. 3; $p>0.8$). These findings suggest that astrocytes, rather than microglia, may be important for generating a radiation-induced neuroinflammatory response in PPAR δ KO mice.

GW0742 does not prevent the WBI-induced decrease in hippocampal neurogenesis

Given that GW0742 prevented the acute radiation-induced increase in the number of activated hippocampal microglia (Fig. 2A) and that brain inflammation has been shown to be detrimental to neurogenesis (14, 15, 31), we hypothesized that GW0742 would prevent a radiation-induced decrease in hippocampal neurogenesis. WBI produced an ~90% decrease in the number of BrdU⁺/NeuN⁺ cells in the GCL/SGZ of both WT (radiation effect: $F_{1,23}=48.00$, $p<0.001$) and KO (radiation effect: $F_{1,27}=31.69$, $p<0.001$) mice at 2 months after WBI (Fig. 4). Dietary GW0742 alone had no effect on the number of BrdU⁺/NeuN⁺ cells in the GCL/SGZ of WT (drug effect: $F_{1,23}=0.59$, $p>0.4$) or KO (drug effect: $F_{1,27}=0.37$, $p>0.5$) mice at 2 months after WBI (Fig. 4). Moreover, dietary GW0742 did not prevent/ameliorate the radiation-induced decrease in neurogenesis in either the irradiated WT (interaction effect: $F_{1,23}=0.48$, $p>0.5$) or KO (interaction effect: $F_{1,27}=0.08$, $p>0.7$) mice (Fig. 4).

GW0742 does not prevent radiation-induced, hippocampal-dependent cognitive impairment

Although there was no effect of either dietary GW0742 or WBI on hippocampal-dependent spatial learning as measured by the latency of WT mice to locate the escape box during the training trials on Days 1-5 at 3 months after a single 10 Gy dose of WBI, a significant radiation-induced decrease in hippocampal-dependent spatial memory ($Z = -2.64$, $p < 0.01$) was measured during the trials on Days 7-11 (Fig. 5A). However, this radiation-induced decrease in hippocampal-dependent memory was not prevented/ameliorated ($Z = -0.04$, $p > 0.9$) by dietary GW0742. No statistically significant effects were measured by the frequency in the tunnel (escape box) zone on the Day 6 (radiation: $F_{1,56} = 0.21$, $p > 0.6$; drug: $F_{1,56} = 2.36$, $p > 0.1$; interaction: $F_{1,56} = 0.21$, $p > 0.6$) or the Day 12 (radiation: Bonferroni $p > 0.05$; drug $F_{1,56} = 1.13$, $p > 0.3$; interaction ($F_{1,56} = 0.30$, $p > 0.6$) probe trial (Fig. 5B,C). Thus, dietary GW0742 was not able to protect against the early delayed cognitive impairment measured in WT mice at 3 months after WBI.

No radiation-induced deficits in hippocampal-dependent spatial learning or memory were measured in WT mice at 6 months after a single 10 Gy dose of WBI using the: i) latency to the escape box (Fig 5D; radiation, $Z = -0.20$, $p > 0.8$; drug, $Z = -0.27$, $p > 0.7$; interaction, $Z = 1.33$, $p > 0.2$) or ii) frequency in the tunnel zone during the Day 6 probe trial (Fig. 5E; radiation: $F_{1,50} = 0.12$, $p > 0.7$); drug: $F_{1,50} = 0.00$, $p > 0.9$; interaction: $F_{1,56} = 0.00$, $p > 0.9$). These results suggest that the cognitive impairment observed 3 months after WBI was a transient early delayed response that did not lead to progressive cognitive impairment at 6 months after WBI. However, at 6 months after irradiation, a significant ($F_{1,53} = 9.40$, $p < 0.01$, Bonferroni $p < 0.05$) reduction in the frequency to the tunnel was measured on the Day 12 probe trial for the mice on dietary GW0742, with or without radiation. Taken together, these results suggest that, if clinical trials with PPAR δ agonists are to be implemented, the drugs

should be used only for short periods of time because long term regimens may have detrimental effects on cognitive function.

Discussion

The main findings of this study were: i) dietary GW0742 prevents the increase in IL-1 β mRNA and ERK phosphorylation levels after WBI; ii) dietary GW0742 prevents the WBI-induced increase in activated hippocampal microglia in WT mice; iii) PPAR δ KO mice do not exhibit a WBI-induced increase in activated hippocampal microglia; iv) WBI decreases the number of astrocytes in WT, but not PPAR δ KO mice; v) dietary GW0742 does not restore hippocampal neurogenesis following WBI; and vi) dietary GW0742 does not prevent early delayed hippocampal-dependent cognitive impairment observed 3 months after WBI.

Microglia have many functions in the brain, including supporting the growth of neurons [32]. In response to various stimuli, such as radiation, microglia become activated and undergo physiological changes, including upregulation of the lysosomal antigen macrophage marker (CD68) and release of proinflammatory factors [33, 34]. Neuroinflammation is associated with radiation-induced brain injury [13, 14, 35]. Thus, preventing/ameliorating the increase in proinflammatory responses following irradiation appears a rationale pharmacologic approach to reducing radiation-induced brain injury. Given the potent anti-inflammatory effects of PPAR δ activation in microglia and astrocytes *in vitro*, we hypothesized that dietary GW0742 would prevent both the WBI-induced increase in markers of neuroinflammation and activated hippocampal microglia [25]. As predicted, dietary GW0742 prevented the radiation-induced increase in i) IL-1 β mRNA (Fig. 1A) and ERK phosphorylation (Fig. 1B), two markers of neuroinflammation, 3 h after WBI; and ii) the number of CD68 $^{+}$ cells in the DG/hilus of WT mice 1 week after WBI (Fig. 2A). Consistent with previous studies in our lab using 129S1/SvImJ mice [19], the number of activated hippocampal microglia returned to the sham-irradiated level by 2 months after WBI (Fig. 2B). Thus, in our WT murine models, hippocampal microglial activation appears to be an early event after WBI. To our knowledge, this is the first demonstration of a role for PPAR δ in modulating inflammatory markers and hippocampal microglial activation after WBI.

GW0742 has been shown to act independently of PPAR δ [30]. We therefore used PPAR δ KO mice to investigate whether PPAR δ is required for the anti-inflammatory properties of GW0742. Surprisingly, in our model, the number of activated microglia was unaltered both 1 week (Fig. 2C) and 2 months (Fig. 2D) after WBI in the DG/hilus of the KO mice. It is unclear whether this results from i) a requirement for PPAR δ to generate an inflammatory response after WBI, or ii) developmental compensatory mechanisms in the KO mice that may impair the brain's ability to mount a normal radiation inflammatory response. Regardless of the underlying mechanism, the lack of microglial activation in the irradiated KO mice precluded our ability to determine whether the anti-inflammatory properties of GW0742 were independent of its action on PPAR δ . In addition to having a different hippocampal microglial response after WBI, PPAR δ KO mice also had a different astrocytic response than WT mice. Although the number of GFAP $^{+}$ cells was decreased significantly in the WT mice 2 months after WBI, it was not in the KO mice (Fig. 3B). These findings are

consistent with unpublished data from our lab suggesting that PPAR δ also regulates the astrocyte response after WBI.

Given that previous studies have demonstrated a negative correlation between activated hippocampal microglia and neurogenesis [15, 17, 19], we hypothesized that dietary GW0742 administration would prevent/ameliorate the WBI-induced decrease in hippocampal neurogenesis. Although we confirmed a significant decrease in hippocampal neurogenesis 2 months after WBI, dietary GW0742 did not prevent/ameliorate this decrease (Fig. 4). Our present findings are in agreement with previous data, suggesting that a simple link between inhibiting hippocampal microglial activation and restoring hippocampal neurogenesis after irradiation is unlikely [36, 37]. Thus, the exact relationship between neuroinflammation, neurogenesis, and cognition remains to be defined. For example, administration of either the angiotensin-converting enzyme (ACE) inhibitor, ramipril, or the angiotensin II type I receptor antagonist (AT₁RA), L-158,809, before, during, and after fWBI prevented or ameliorated cognitive impairment [36, 37]. However, neither renin angiotensin system (RAS) blocker modulated radiation-induced hippocampal neuroinflammation or restored hippocampal neurogenesis. Taken together, although radiation-induced cognitive impairment has been associated with increased hippocampal microglial activation and decreased hippocampal neurogenesis (14, 18, 38), cognitive defects can be prevented/ameliorated without concomitantly modulating hippocampal microglial activation or hippocampal neurogenesis.

Clinically, radiation-induced brain injury is characterized by progressive cognitive impairment [2, 6-10]. One limitation of our studies is that the cognitive impairment that we observed in WT mice 3 months after WBI was no longer present at 6 months, suggesting that the impairment was a transient early delayed response, rather than a progressive cognitive impairment. Of note, we and others in the Radiation-Induced Brain Injury and Treatment group at Wake Forest have also been unsuccessful in detecting hippocampal-dependent cognitive impairment in young adult male rats administered 40 Gy of fWBI [unpublished data]. However, using the Novel Object Recognition (NOR) task that assesses perirhinal cortex-dependent cognitive function, we have measured progressive cognitive impairment 6 months to 1 year in young adult male rats after fWBI [37, 39] and prevented it with the PPAR δ agonist, pioglitazone, in the diet [39]. Although in the present study, dietary GW0742 did not protect against early delayed cognitive impairment in WT mice, it is possible that the mechanisms underlying the early delayed cognitive impairment measured by the Barnes maze task are different from those contributing to progressive late delayed cognitive impairment measured by the NOR task. Consequently, future studies should examine if dietary GW0742 can prevent/ameliorate radiation-induced late delayed perirhinal cortex-dependent cognitive impairment in young adult male rats using the NOR task.

In summary, dietary GW0742 prevents the WBI-induced increases in IL-1 β mRNA, ERK phosphorylation, and hippocampal microglial activation in WT mice. However, dietary GW0742 does not restore the WBI-induced decrease in hippocampal neurogenesis, or prevent early delayed hippocampal-dependent cognitive impairment in WT mice. Nevertheless, the mechanism(s) by which PPAR δ agonists prevent these radiation-induced

neuroinflammatory responses and its relationship to radiation-induced cognitive impairment remain elusive.

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Figure 1. GW0742 administration prevents the WBI-induced acute increase in IL-1 β and p-ERK
A: A significant increase in IL-1 β mRNA was measured 3 h after a single 10 Gy dose of WBI in WT mice that was prevented by dietary GW0742. **B:** A significant increase in p-ERK was measured 3 h after a single 10 Gy dose of WBI in WT mice that was prevented by dietary GW0742. Data are presented as the Mean $\pm$ SD; n=3/group. * Bonferroni p<0.05; ** Bonferroni p<0.01.

Figure 2. The effect of WBI and dietary GW0742 on hippocampal microglia activation is different in WT and PPARδ KO mice

A significant increase in the number of activated hippocampal microglia (CD68⁺ cells) was measured 1 week after a single 10 Gy dose of WBI in WT that was prevented by dietary GW0742 (A). No increase in the number of activated hippocampal microglia was detectable in WT mice 2 months after WBI (B). PPARδ KO mice had no detectable increase in activated hippocampal microglia at either 1 week (C) or 2 months (D) after a single 10 Gy dose of WBI. Data are presented as the Mean ± SD; n=3/group. ** Bonferroni p<0.01; *** Bonferroni p<0.001.

Figure 3. WBI decreases the number of astrocytes (GFAP+ cells) in WT, but not in PPAR δ KO mice

A: Left panel: low power view of the DG/hilus containing stained astrocytes; Right panel: high power view of the DG/hilus used for counting the astrocytes (white arrows). **B:** A significant decrease in the number of hippocampal astrocytes was measured in the DG/hilus of WT mice at 2 months after a single 10 Gy dose of WBI. Unirradiated PPAR δ KO mice had a reduced number of hippocampal astrocytes that were not reduced further by WBI. Data are presented as the Mean $\pm$ SD; n=3/group. * Bonferroni $p < 0.05$.

A **B**

Figure 4. Dietary GW0742 does not prevent the WBI-induced decrease in hippocampal neurogenesis

A: Representative confocal image of BrdU/NeuN double labeling. Left panel: Hippocampal neurons labeled with the mouse α -NeuN antibody (green); Middle panel: Proliferating hippocampal cells labeled with the mouse α -BrdU antibody (red); Right panel: Merged image. Yellow arrow identifies a newborn neuron; white arrow identifies a non-neuronal proliferating cell. **B:** An ~90% decrease in the number of newborn hippocampal neurons (BrdU⁺/NeuN⁺ cells) was measured in both WT and PPAR δ KO mice at 2 months after a single 10 Gy dose of WBI that was not prevented by dietary GW0742. Data are presented as the Mean $\pm$ SEM; n=6-8/group; ** Bonferroni $p < 0.01$, *** Bonferroni $p < 0.001$.

3 Months After WBI

6 Months After WBI

Figure 5. Dietary GW0742 does not prevent/ameliorate early delayed impairment of hippocampal-dependent spatial memory

Three months after a single 10 Gy dose of WBI, WT mice had an increased in the latency to the escape box that was not prevented by dietary GW0742 (A). WT mice exhibited no change in the frequency in the tunnel zone on the Day 6 (B) and Day 12 (C) probe trials, with or without, dietary GW0742 at 3 months after WBI. WT mice exhibited no change in both the latency to the escape box (D) and the frequency in the tunnel zone on the Day 6 probe trial (E), with and without, dietary GW0742 at 6 months after a single 10 Gy dose of WBI. However, on the Day 12 probe trial (F), WT mice on dietary GW0742 exhibited a significant decrease in the frequency in the tunnel zone, with and with WBI. Data are presented as Mean $\pm$ SEM; n=15/group. * p<0.05.