RADIATION-INDUCED INFLAMMATORY MARKERS OF BRAIN INJURY ARE MODULATED BY PPARδ ACTIVATION IN VITRO AND IN VIVO

By

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<tr>
<td>8-OHdG</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AP-1</td>
<td>activator protein-1</td>
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<td>BBB</td>
<td>blood-brain barrier</td>
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<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>Cox-2</td>
<td>cycloxygenase-2</td>
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<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>cycle threshold</td>
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<tr>
<td>DCF</td>
<td>dichlorofluorescein diacetate</td>
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<td>DG</td>
<td>dentate gyrus</td>
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<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<td>ERK</td>
<td>extracellular signal-related kinase 1/2</td>
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<td>GCL</td>
<td>granule cell layer</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>Gy</td>
<td>Gray</td>
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<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
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<tr>
<td>HNE</td>
<td>4-hydroxynonenal</td>
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<td>IL-1β</td>
<td>interleukin-1 beta</td>
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<td>IkBα</td>
<td>inhibitor of NF-κB alpha</td>
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<tr>
<td>KO</td>
<td>knock-out</td>
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<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<td>MEK1/2</td>
<td>mitogen-activated protein kinase kinase 1/2</td>
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<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<td>NeuN</td>
<td>neuronal nuclei</td>
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<tr>
<td>PKCα</td>
<td>protein kinase C alpha</td>
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<tr>
<td>PLA₂</td>
<td>phospholipase A₂</td>
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<tr>
<td>PPARδ</td>
<td>peroxisomal proliferator-activated receptor delta</td>
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<tr>
<td>PPRE</td>
<td>PPAR response element</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RT-PCR</td>
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<td>sub-granular zone</td>
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<td>tumor necrosis factor alpha</td>
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<td>WBI</td>
<td>whole brain irradiation</td>
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ABSTRACT

Radiation-induced inflammatory markers of brain injury are modulated by PPARδ activation *in vitro* and *in vivo*

Dissertation under the direction of Mike E. Robbins, Ph.D., Professor and Section Head of Radiation Biology, Department of Radiation Oncology

As a result of improvements in cancer therapy and health care, the population of long-term cancer survivors is growing. For these approximately 12 million long-term cancer survivors, brain metastases are a significant risk. Fractionated partial or whole-brain irradiation (fWBI) is often required to treat both primary and metastatic brain cancer. Radiation-induced normal tissue injury, including progressive cognitive impairment, however, can significantly affect the well-being of the approximately 200,000 patients who receive these treatments each year. Recent reports indicate that radiation-induced brain injury is associated with chronic inflammatory and oxidative stress responses, as well as increased microglial activation in the brain. Anti-inflammatory drugs may, therefore, be a beneficial therapy to mitigate radiation-induced brain injury. We hypothesized that activation of peroxisomal proliferator activated receptor delta (PPARδ) would prevent or ameliorate radiation-induced brain injury, including cognitive impairment, in part, by alleviating inflammatory responses in microglia.

For our *in vitro* studies, we hypothesized that PPARδ activation would prevent the radiation-induced inflammatory response in microglia following irradiation. Incubating BV-2 murine microglial cells with the PPARδ agonist, L-165041, prevented the radiation-induced increase in: i) intracellular ROS generation, ii) Cox-2 and MCP-1 expression, and iii) IL-1β and TNF-α message levels. This occurred, in part, through PPARδ-mediated modulation of stress activated kinases and proinflammatory transcription factors. PPARδ inhibited NF-κB via transrepression by physically interacting with the p65 subunit, and prevented activation of the PKCα/MEK1/2/ERK1/2/AP-1 pathway by inhibiting the radiation-induced increase in intracellular
ROS generation. These data support the hypothesis that PPARδ activation can modulate the radiation-induced oxidative stress and inflammatory responses in microglia in vitro.

To extend our in vitro findings in vivo, we investigated whether administration of the peroxisomal proliferator-activated receptor (PPAR)δ agonist, GW0742, prevented radiation-induced brain injury in C57Bl/6 WT mice. Our data demonstrate that GW0742 prevented the radiation-induced increase in the number of activated microglia (CD68+ cells) in wild-type (WT) mice 1 week following 10 Gy WBI. Furthermore, GW0742 inhibited the WBI-induced increase in IL-1β message levels and ERK phosphorylation observed 3 h post-irradiation. In contrast, GW0742 administration failed to modulate the radiation-induced decrease in hippocampal neurogenesis (NeuN+/BrdU+ cells) determined 2 months after irradiation, or mitigate hippocampal-dependent spatial memory impairment observed 3 months post-irradiation using the Barnes Maze task. We used PPARδ knockout (KO) mice to examine if the effects of GW0742 are PPARδ-dependent. Unexpectedly, PPARδ KO mice exhibited a differential response following WBI compared to WT mice; therefore, we were unable to make mechanistic conclusions about GW0742. KO mice do not exhibit a WBI-induced increase in activated microglia; however, they appeared to display a pronounced astrocytic response. In particular, PPARδ KO but not WT mice displayed increased GFAP message levels 2 months after WBI. Additionally, the number of GFAP+ cells was reduced significantly in the WT mice 2 months after WBI, but it was not in the PPARδ KO mice. These results demonstrate that: i) GW0742 prevents the radiation-induced increase in microglial activation and inflammatory markers, and ii) WT and PPARδ KO mice have a differential response to WBI.
I.1 Treatment and Management of Brain Metastasis

In 2012, 1.6 million new cancer cases are expected to be diagnosed in the United States alone, of which approximately 30% will develop brain metastases. Due to an aging population, better systemic treatment, and improved imaging modalities to detect metastases in asymptomatic patients, the incidences of brain metastasis are increasing [1]. Approximately half of all brain metastases arise from lung cancer [1]. Breast cancer, unknown primary, and melanoma are also frequently associated with brain metastases [1-3].

The prognosis for patients with brain metastases is poor. The median survival time without treatment is approximately 1 month, and with treatment is 4-6 months [4, 5]. The treatment modalities for brain metastases include neurosurgery, stereotactic radiosurgery (SRS), and fractionated partial or whole-brain irradiation (fWBI). Since many primary tumors can vary in their sensitivities to fWBI, surgical resection is a treatment option for patients with metastases of unknown histological type [6]. For example, renal cell carcinoma and melanoma are very resistant to fWBI, while small cell lung cancer is very radiosensitive [6]. Surgical resection may also be used for patients who have previously received cranial irradiation. A benefit of surgical resection is that it offers relief of symptoms caused by intracranial pressure [6]. SRS is generally used to treat patients with 1-3 metastases and targets tissue up to 3 cm in maximum diameter. An advantage of using SRS is that it can treat lesions that are inaccessible surgically [5, 6].

fWBI is the only treatment option for patients who present with brain metastases that are too large, numerous, or dispersed to treat with surgery or SRS [1]. Given that approximately 70% of patients present with multiple brain metastasis, fWBI is the primary treatment modality for brain metastasis [1, 5]. The use of fWBI can decrease the recurrence rate, attenuate
neurological symptoms, and improve the quality of life of patients [7]. The Radiation Therapy Oncology Group (RTOG) has documented that over 60% of patients in randomized controlled studies had a complete or partial response to fWBI. Although studies indicate that differences in dose, timing, and fractionation do not alter significantly the median survival time of patients treated with fWBI, the most common fWBI regimen in the US is 30 Gy in ten 3 Gy fractions over 2 weeks [1].

fWBI also is given routinely as an adjuvant therapy to patients who receive SRS; however, since fWBI has been associated with long-term neurotoxicity, several studies have investigated the effectiveness of SRS administered alone or in combination with fWBI [1]. Three randomized control trials have demonstrated that patients administered fWBI+SRS had significantly improved local tumor control compared to patients treated with SRS alone. Better intracranial tumor control by fWBI+SRS is associated with an improvement of both executive function and fine motor coordination in patients [8, 9]. Thus, fWBI remains the primary treatment modality of brain metastases.

I.2 Radiation-Induced Brain Injury

A current challenge of using fWBI to treat brain metastasis is that normal brain tissue, in addition to the tumor, is irradiated. The radiation dose that can be delivered to the tumor, therefore, is limited by the risk of toxicity to the surrounding normal brain tissue. Radiation-induced brain injury is characterized as acute, early delayed, or late delayed injury based upon time of expression [10]. Acute injury occurs days to weeks following irradiation and is characterized by headache, drowsiness, vomiting, and nausea. These symptoms are rare under the current radiotherapy regimens and can be treated with corticosteroids. Early delayed injury presents itself 1 to 6 months post-irradiation and it is distinguished by transient demyelination, somnolence, attention deficits, and short-term memory loss. Although acute and
early delayed injury can result in severe damage, they are reversible and often spontaneously resolve themselves [10].

In contrast to acute and early delayed injury, late delayed injury, which is observed more than 6 months post-irradiation, is often irreversible and progressive. Late delayed effects may include demyelination, vascular abnormalities, white matter necrosis, and cognitive impairment [11, 12]. Approximately 30% of long-term brain tumor survivors treated with fWBI will develop cognitive deficits ranging from mild lassitude to severe dementia [13-15]. The incidence and severity of the cognitive deficits increase with prolonged survival time. With better systemic agents improving the life expectancy of patients with cancer, patients with metastatic brain disease are living longer; consequently, they are at a higher risk for developing a radiation-induced cognitive impairment. Not surprisingly, a patient’s quality of life (QOL) is negatively affected by radiation-induced cognitive impairment [13-15]. This diminished QOL has become an important concern for these long-term survivors of brain irradiation, and is recognized as one of the most important measurements of brain tumor therapy outcomes in clinical trials, second only to survival [16-18]. Although studies have investigated whether modifying the delivery of fWBI or pharmacological approaches can modulate cognitive impairment, there are no proven long-term treatments for radiation-induced cognitive deficits. It is, therefore, important to investigate new therapeutic approaches [18].

Patients who receive fWBI often develop deficits in hippocampal-dependent learning and memory, including spatial information processing. These deficits have been hypothesized to be due to a depletion of the radiosensitive neural precursor cells in the dentate gyrus of the hippocampus, which is one of only two sites of neurogenesis in the human brain [19-23]. Therefore, one approach to minimize neurotoxicity is to deliver fWBI with hippocampal avoidance [24-27]. Given that 3% of brain metastases are distributed within a 5-mm margin around the hippocampus, conformal avoidance of the hippocampus during fWBI represents an acceptable risk of loss of tumor control [28]. It is unknown, however, if hippocampal avoidance
can delay the onset of cognitive impairment. A concern of this approach is that WBI-induced cognitive impairment is both hippocampal-dependent and –independent. In particular, patients who receive WBI often present with deficits in executive function, which is a prefrontal cortex-dependent cognitive function [1]. Thus, other approaches to minimize neurotoxicity need to be examined.

Clinical trials have investigated whether pharmacological approaches can ameliorate and/or prevent cognitive impairment [24, 30, 31]. In particular, phase II clinical trials have demonstrated that adult brain cancer patients administered donepezil, an acetylcholinesterase inhibitor, had significant improvements in QOL, mood, and cognitive function, including improved attention, concentration, and memory following the 24-week study [30]. Alpha-tocopherol (vitamin E) administered for 1 year to patients with temporal lobe radionecrosis has also been shown in a phase II clinical trial to significantly improve cognitive function, memory, and executive function [31].

I.3 Radiation-Induced Oxidative Stress and Inflammation

Classically, radiation-induced late normal tissue injury was thought to be caused by a reduction in the number of surviving clonogens of the parenchymal or vascular target cell population [11, 32]. Late effects were, therefore, thought to be progressive and untreatable. Recent data, however, suggest that this view of radiation-induced brain injury is over simplistic. Instead, late effects are thought to involve modulation of various signaling pathways in a variety of cell types; in the brain these include astrocytes, endothelial cells, microglia, neurons, and oligodendrocytes [19, 33-35]. This complex response is thought to include activation of stress-activated kinases and transcription factors, which contribute to increased oxidative stress and inflammation [36-38]. This model of radiation-induced brain injury suggests that late effects, including cognitive impairment, can be mitigated therapeutically by modulating altered signaling
pathways that contribute to normal tissue injury, including oxidative stress and inflammatory responses.

Oxidative stress is the result of an imbalance in cells between i) the levels of reactive oxygen species (ROS) and ii) the anti-oxidant defenses that help prevent the reactive intermediates from damaging cells and tissues. ROS are both free radicals, including superoxide (O$_2^•-$), nitric oxide (NO), and the hydroxyl radical (OH$^•$); and molecular species, such as hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^•$). ROS are important cell signaling mediators; however, excessive amounts of ROS can damage cellular DNA, proteins, and lipids [39]. The brain is particularly susceptible to oxidative damage since it: i) consumes 20% of the oxygen in the body, which leads to increased mitochondrial O$_2^{•}$; ii) contains a high level of iron contributing to free radical formation; iii) contains a high level of unsaturated fatty acids that can be peroxidized by ROS; and iv) contains a low level of anti-oxidant enzymes, including catalase, SOD, and glutathione peroxidase [40-43]. Studies in the rodent brain indicate that irradiation leads to an increase in oxidative stress. Increased levels of 4-hydroxynonenal (HNE) and 8-hydroxy-2’deoxyguanosine (8-OHdG), products of lipid peroxidation and DNA oxidation, have been identified in the hippocampi of adult male mice 1 month following irradiation [44]. Furthermore, a time-dependent increase in nitrotyrosine, indicative of nitroative stress, has been observed in the subventricular zone (SVZ) of postnatal day 8 rats or postnatal day 10 mice irradiated with a single dose of 4-12 Gy of 4 MV X-rays [45]. In addition to their direct effects on DNA, protein, and lipids, ROS can act as second messengers to induce inflammation [43].

Although it was once believed that the brain was not susceptible to inflammation, it is now recognized that inflammatory responses do occur and may contribute to radiation-induced brain injury [46]. Studies performed in vivo indicate that there is an increase in the expression of proinflammatory mediators, including TNF-α, IL-1β, ICAM-1, Cox-2, and NF-κB within hours of irradiating the rodent brain [20, 47, 48]. Maximal NF-κB activation, indicated by NF-κB-dependent bioluminescent intensity in transgenic mice carrying a NF-κB-driven luciferase gene,
has been observed 3 h following a single dose of 8.5 Gy total-body irradiation [49]. Furthermore, a chronic elevation of TNF-α has been observed in the mouse brain 6 months following irradiation with a single dose of 25 Gy [47].

Microglia, which are termed the macrophage of the brain, are considered to be one of the key mediators of neuroinflammation [50-53]. They form approximately 10% of the total glial cell population in the central nervous system (CNS), and are the primary immune cells of the brain [54]. In an uninjured brain, microglia exist as resting or ramified cells with an elongated soma containing processes. Ramified microglia actively monitor the CNS to ensure that the brain is maintaining homeostasis [51]. Microglia also secrete neuronal growth factor (NGF), a neurotrophic factor, to promote neuronal survival [55]. Following injury to the brain, microglia become activated and undergo physiological changes. Microglial activation is a process characterized by rounding of the cell body, retraction of cell processes, proliferation, and an increased production of cytokines, chemokines, and reactive oxygen species (ROS) [19, 50-53]. Although microglial activation plays an important role in the phagocytosis of dead cells, sustained activation is thought to contribute to a chronic proinflammatory state in the brain [51]. Microglial activation has been observed both 1 week and 2 months following irradiation of the mouse and rat brain, respectively [19, 56]. Furthermore, irradiating microglial cells in vitro induces an increase in proinflammatory mediators, such as the cytokines, TNF-α and IL-1β, and the chemokines, MCP-1 and ICAM-1 [57-59].

The radiation-induced increase in microglial activation in the hippocampus is thought to alter the microenvironment of the neurogenic niche [19]. Neurogenesis is a complex process that results in the production of new neurons. Neurogenesis begins with the proliferation of progenitor cells in the SGZ, is followed by the commitment of cells to a neuronal phenotype, and ends with a functional neuron integrated into the GCL [60]. Irradiation reduces the proliferative capacity of neuronal precursor cells in vitro, and leads to a significant decrease in the number of immature and mature newborn neurons in the DG of rodents in vivo [19, 56, 61]. As previously
stated, the WBI-induced decrease in neurogenesis is associated with defects in hippocampal-dependent learning and memory [27]. Studies in rodents indicate that there is a negative correlation between microglial activation and hippocampal neurogenesis following irradiation [62]. Furthermore, anti-inflammatory drugs have been shown to inhibit the radiation-induced increase in microglial activation, and concomitantly, ameliorate/prevent the radiation-induced decrease in hippocampal neurogenesis [62, 63]. Altered neurogenesis as a result of oxidative stress and/or inflammation, therefore, appears to be one of the mechanisms underlying radiation-induced brain injury. The radiation-induced increase in oxidative stress and proinflammatory responses produced by microglia may also alter the functions of the pre-existing neurons [59]. In particular, the radiation-induced increase in ROS generation and inflammatory cytokines, such as IL-1B, are thought to induce defects in the long-term potentiation (LTP) in hippocampal slices. LTP is a measure of signal transmission between two neurons that is thought to underlie learning and memory [64-71]. Lonergan et al. demonstrated that eicosapentaenic acid, a poly-unsaturated fatty acid with anti-inflammatory properties, restored the altered LTP in whole-body irradiated rats [64]. Taken together, these findings provide a strong rationale for investigating anti-inflammatory therapies to mitigate radiation-induced late effects in the brain.

I.4 Peroxisomal Proliferator-Activated Receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily. Three closely related subtypes have been identified—PPARα (NR1C1), PPARδ (NR1C2, PPARβ), and PPARγ (NR1C3) [72]. They are ligand-activated transcription factors that heterodimerize with the retinoid X receptor and regulate transcription by binding to PPAR response elements (PPRE) [73, 74]. PPREs, located in the enhancer regions of genes, consist of an AGGTCA hexameric direct repeat separated by one or two nucleotides [74]. PPARs are activated by a broad range of natural and synthetic ligands and
regulate a wide range of cellular processes, including inflammation, proliferation, differentiation, metabolism, and energy homeostasis. PPAR subtypes are encoded by different genes, have unique tissue distributions, and exhibit overlapping and differential functions.

PPARα is expressed predominantly in metabolically active tissues, such as the liver, kidney, heart, brown adipose tissue, muscle, and small and large intestines [75-77]. Natural ligands of PPARα include saturated and unsaturated long-chain fatty acids and their eicosanoid derivatives [78]. Fibrates such as fenofibrate, gemfibrozil, and clofibrate are marketed PPARα agonists that are used for the treatment of hypertriglyceridemia [79]. Additionally, in clinical trials, PPARα agonists appear to be protective for the treatment of cardiovascular diseases [80].

In contrast to PPARα and PPARγ, PPARδ is thought to be ubiquitously expressed, with high expression in the intestinal epithelium, liver, and keratinocytes [81]. Natural ligands of PPARδ include long chain fatty acids, triglycerides, prostacyclin, prostaglandin A₃, and all trans retinoic acid [82]. Thus far, the only PPARδ agonist in clinical trials is GW501516, which is being investigated for the treatment of dyslipidemia. The PPARδ agonist increased high-density lipoproteins in healthy individuals and decreased fasting plasma triglycerides, apolipoprotein B, low-density lipoproteins, cholesterol, and insulin in moderately obese individuals [83, 84].

There are two isoforms of PPARγ—PPARγ1 and PPARγ2. PPARγ1 is expressed at high levels in the adipose tissue and also at low levels in many other tissues, such as immune cells and the brain [76, 85]. In contrast, PPARγ2 is expressed only in the adipose tissue [86]. PPARγ is activated by endogenous eicosanoids 9- and 13-hydroxyoctadecadienoic acids [87]. Thiazolidinediones (TZD) are insulin-sensitizing drugs that activate PPARγ [88]. Pioglitazone and rosiglitazone are two TZDs currently used for the treatment of type-2 diabetes [89]. Clinical trials are also assessing the anti-atherosclerotic efficacy of TZDs. Early clinical results indicate that TZD treatment prevents the progression of carotid artery plaques and reduces plasma levels of inflammatory biomarkers for cardiovascular disease [79].
Despite sharing the same PPRE, research has demonstrated that the PPAR transcriptomes are subtype specific by ectopically expressing individual PPAR subtypes and treating cells with specific agonists. Studies, therefore, have focused on understanding how the individual PPARs can confer subtype specificity beyond the scope of tissue expression and ligand specificity [90-92]. There is particular interest in understanding which domains are involved in enhancing or preventing target gene transcription. Similar to most nuclear receptors, the protein structure of PPARs consists of five domains designated A/B, C, D, E, and F (shown below). The C- and E-domains share a high degree of sequence and structural homology between the PPAR subtypes, approximately 80-90% and 60-70%, respectively. In contrast, the A/B- and D-domains are less conserved among the PPAR subtypes sharing only 20-30% homology [87, 93].

![Diagram of PPAR domains]

The N-terminal A/B-domain contains the ligand-independent activation function-1 (AF-1) region, which has a low level of basal transcription activity [94]. Of the four PPAR domains, the A/B-domain is the least conserved. It has, therefore, been hypothesized that PPAR subtype-specificity is largely maintained by this domain. Studies have used PPAR chimeric proteins in which the A/B-domain is exchanged between the subtypes to demonstrate that subtype-specificity is maintained by the A/B-domain [95, 98]. It has been proposed that different cofactors can bind to the different surfaces of the poorly structured A/B-domains causing the domains to fold into different conformations. These conformations may have differential transcriptional activity or DNA specificity [99]. It has also been suggested that post-translational modifications of the A/B-domain may induce a secondary structure, which enhances binding of cofactors to the N-terminal domain [100].
Next to the A/B-domain is the DNA-binding C-domain, which contains two highly conserved zinc finger motifs that are capable of binding to PPREs. Following the C-domain is the D-domain or hinge region. The C-terminal E-domain contains the ligand-dependent activation function-2 (AF-2) region. The AF-2 dependent transcriptional activation of PPARs is mediated by ligand-induced conformational changes in the ligand-binding domain (LBD). Of note, PPARδ has a smaller ligand-binding pocket than PPARα or PPARγ, and therefore, may bind to fewer ligands. This may explain why fewer PPARδ agonists have been identified. Also of interest, the ligand-binding pocket of PPARα is more lipophilic than the other PPAR receptors [101]. The ligand-dependent activation of PPARs is important to stabilize the LBD, which allows the binding of coactivators, such as histone acetylases to LXXLL consensus sites [102]. In the absence of ligand binding, transcription is inhibited by binding of PPARs to co-repressors, such as histone deacetylases [95]. Corepressors cannot fit within the LBD but can bind to surface LXXXIXX sites, which partially overlap with the coactivator-binding sites [87, 95]. These cofactors are required to remodel the chromatin to promote or repress transcription and may also influence the subtype-specific transcriptional response.

### I.5 PPARδ-Mediated Modulation of Inflammation

The anti-inflammatory and anti-oxidant properties of PPARδ agonists have been characterized in multiple cell types [82, 103-110]. PPARδ mediates many of its anti-inflammatory effects by: i) preventing activation of NF-κB, ii) releasing or inducing the co-repressor B-cell lymphoma protein-6 (Bcl-6), iii) inhibiting stress-activated kinases, iv) increasing the expression of anti-inflammatory factors, and v) decreasing oxidative stress [75, 106-113].

PPARδ has been shown to modulate inflammation by negatively regulating the proinflammatory transcription factor NF-κB. There are several mechanisms by which PPARδ can modulate NF-κB, including: i) reducing p65 nuclear translocation, ii) decreasing p65 acetylation, and iii) interacting directly with p65. The PPARδ agonist, GW0742, attenuated
myocardial reperfusion injury in rats, in part, by decreasing the nuclear translocation of p65 [114]. Additionally, in endothelial cells, the PPARδ agonist, L-165041, reduced VCAM-1 and MCP-1 expression by decreasing the nuclear translocation of p65 [115]. The PPARδ agonist, GW501516, inhibited TNF-α-induced inflammation in human keratinocytes by preventing p65 acetylation. Acetylation of p65 is regulated by the transcriptional co-activator p300; reduced acetylation of p65 decreases DNA binding and activation of NF-κB [116]. In cardiomyocytes, L-165041 prevented LPS-induced NF-κB activation and MCP-1 expression via transrepression by direct binding of PPARδ to the p65 subunit of NF-κB [106].

Transrepression of inflammatory mediators by PPARδ also is mediated by the PPARδ ligand-induced release of the transcriptional repressor, BCL-6. In macrophages, GW0742 reduced LPS-induced TNF-α by: i) displacing the p65 subunit of NF-κB from the κB elements of the TNF-α promoter, and ii) recruiting Bcl-6 to the TNF-α promoter [117]. PPARδ also has been shown to reduce inflammation in macrophages by displacing Bcl-6 from PPARδ to the promoter regions of MCP-1 [110]. In endothelial cells, GW0742 inhibited inflammation by relocating Bcl-6 to the promoter regions of VCAM-1 [109]. Activation of PPARδ also can induce increased expression of Bcl-6. In particular, increased expression of Bcl-6 following GW0742 administration has been associated with the attenuation of AngII-accelerated atherosclerosis in mice [109].

Studies also have demonstrated that PPARδ activation inhibits proinflammatory responses via decreased activation of the stress-activated MAP kinases. In macrophages, GW0742 ameliorated Angiotensin (AngII)-induced inflammation by decreasing activation of the MAP kinases, p38 and ERK1/2 [109]. GW501516 inhibited IL-6-induced inflammation in adipocytes via decreased phosphorylation of ERK1/2 [118]. Additionally, GW0742 reduced bleomycin-induced lung inflammation in mice, in part, via decreased ERK phosphorylation [119].

In addition to inhibiting the expression of proinflammatory mediators, PPARδ activation can increase the expression of anti-inflammatory mediators. In vascular smooth muscle cells,
GW501516 attenuated IL-1β-induced MCP-1 expression by increasing the transcription and expression of TGF-β [112]. Activation of PPARδ in macrophages attenuated AngII-accelerated atherosclerosis, in part, by inducing the anti-inflammatory mediators, regulator of G-protein signalling (RGS)-4 and RGS5 [109-111]. In addition, PPARδ activation by GW501516 or L-165041 increased the expression of the anti-inflammatory cytokines, IL-4 and IL-10, in mice with experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis [120].

Additional studies indicate that PPARδ regulates oxidative stress by: i) activating transcription of antioxidant genes; ii) enhancing anti-oxidant enzyme activity; and iii) increasing expression of enzymes involved in the detoxification of lipid peroxidation [113, 114]. In endothelial cells, GW0742 and GW501516 decreased ROS production, and upregulated the expression of the antioxidant genes, superoxide dismutase 1, catalase, and thioredoxin [118]. GW501516 also has been shown to induce an increase in heme oxygenase-1 enzyme activity in endothelial cells, which protects cells against cellular stress induced by H$_2$O$_2$ [121]. Furthermore, PPARδ activation in hepatocytes increased the expression of detoxification enzymes involved in 4-HNE metabolism, such as Aldehyde dehydrogenase 3a1 (Aldh3a1), Glutathione S-transferase M3 (Gstm3), and Glutathione S-transferase omega-1 (Gsto1).

I.6 PPARδ in the Central Nervous System

Each PPAR subtype has been identified in the rodent CNS; however, PPARδ is the only subtype ubiquitously expressed in the adult rodent brain and spinal cord [85, 122-124]. In rats, PPARδ begins to be expressed at midgestation, reaching peak expression in all neural tissues in the late stage embryonic brain, suggesting that it may play a role in cell differentiation in the CNS [76]. In situ hybridization and immunolocalization studies have demonstrated that high levels of PPARδ are found in the hippocampus, telencephalic cortex, and the cerebellar cortex of rats [76, 85, 125]. Other studies in the rat brain indicate that PPARδ mRNA is highly expressed in the thalamic nuclei [120]. In the rat spinal cord, PPARδ is the most abundant
PPAR subtype; it is expressed in every cell layer and highly expressed in the lamina II and lamina IX layers [82]. Rat brain endothelial cells also express PPARδ mRNA, suggesting a possible role in blood brain barrier (BBB) maintenance [126]. PPARδ also is widely expressed in the mouse brain, with high levels in the entorhinal cortex, hypothalamus, hippocampus, and corpus callosum. Localization studies identified PPARδ mRNA and protein in mouse neurons and oligodendrocytes, but not in astrocytes [124]. However, in cell culture, PPARδ expression has been documented in mouse and rat cortical astrocytes as well as in rat cerebral astrocytes [127, 128]. Results from our own laboratory demonstrate that the receptor is expressed in microglia. Taken together, PPARδ expression has been observed in all the major cell types within the CNS, including: astrocytes, endothelial cells, microglia, neurons, and oligodendrocytes.

Despite being the predominant subtype in the CNS, data regarding the neuroprotective efficacy of PPARδ agonists are limited compared to PPARα or PPARγ agonists [85]. This likely reflects the fact that PPARδ-specific agonists and antagonists have become available only recently. Several studies have used the synthetic PPARδ-specific agonists, L-165041, L-160,043, GW0742, or GW501516, to explore the functional activities of the receptor [110, 129-131]. Recent studies assessing the neuroprotective efficacy of synthetic PPARδ agonists indicate that they may ameliorate clinical symptoms and reduce the severity of a variety of acute and chronic CNS pathologies, in large part, by modulation of the oxidative stress and inflammatory responses associated with the diseases. For example, L-160,043 prevented Streptozotocin (STZ)-induced progressive neurodegeneration that corresponds to the clinical symptoms of Alzheimer’s disease, and ameliorated cognitive impairment as evaluated by the Morris Water Maze task in mice. The effects of L-160,043 were attributed, in part, to attenuated oxidative stress. In particular, L-160,043 reduced immunoreactivity of the oxidative stress markers, 8-OHdG and HNE, relative to STZ treated animals [131]. Since elevated levels of 8-OHdG and HNE have been detected in AD brains, PPARδ agonists may be efficacious
antioxidant agents to ameliorate oxidative stress associated with AD [132, 133]. Furthermore, PPARδ activation can reduce inflammatory mediators associated with various CNS disorders. For example, GW501516 or L-165041 attenuated symptoms of multiple sclerosis in mice by decreasing the expression of the proinflammatory cytokines, IL-2 and IL-23, and increasing the expression of the anti-inflammatory cytokines, IL-4 and IL-10. Reduced production of IFNγ and IL-17 by T helper type 1 (Th1) and Th17 cells was also observed in mice treated with GW501516 or L-165041 [120].

Recent reports suggest that PPARδ may have additional protective effects against the progression of CNS disorders, such as promoting cell survival and maintaining cell-matrix adhesions [129, 130, 134]. In particular, GW501516 significantly reduced cerebral vascular endothelial cell degeneration in an oxygen-glucose deprivation mouse model. GW501516 protected against vascular cell death by inhibiting the expression of miR-15a, which directly regulates the anti-apoptotic protein Bcl-2. This resulted in increased Bcl-2 protein expression, reduced Golgi fragmentation, and decreased caspase-3 activity, which in turn reduced cerebrovascular permeability and infarct volume in vivo [135]. PPARδ may also protect against ischemia-induced parenchymal and vascular damage by decreasing activity of matrix metalloproteinases (MMPs), enzymes that degrade structural proteins in the extracellular matrix. In particular, treatment of vascular smooth muscle cells (VSMC) with the PPARδ agonist, GW501516, reduced MMP-9 enzymatic activity 24 h after oxygen-glucose deprivation. Furthermore, VSMC-selective PPARδ knockout mice exhibited increased MMP-9 expression following middle cerebral artery occlusion, and inhibition of MMP-9 using shRNA reduced infarct size and vascular permeability in these mice [130].

To date, the neuroprotective benefits of PPARδ agonists have been observed in models of stroke, multiple sclerosis, Alzheimer’s disease, Parkinson’s disease, and spinal cord injury [129, 136-142]. The role of PPARδ in the modulation of radiation-induced brain injury is unknown. Previous studies from our lab have demonstrated that both PPARα and PPARγ
agonists can ameliorate or prevent the severity of radiation-induced brain injury. In particular, the PPARγ agonist, pioglitazone, ameliorated radiation-induced cognitive impairment when administered prior to, during, and for 4 or 54 weeks after WBI of young adult male rats [38]. Although these results are promising, there are some concerns with using pioglitazone in the clinic, including an increased risk of i) weight gain and ii) myocardial infarctions [44]. Our lab also has demonstrated that the PPARα agonists, fenofibrate and GW7647, prevent radiation-induced proinflammatory responses in microglia in vitro. Specifically, fenofibrate and GW7647 prevented the radiation-induced increase in: i) Cox-2 protein, ii) NF-κB and AP-1 activation, and iii) IL-1β and TNF-α message levels in BV-2 murine microglia cells [57]. Furthermore, fenofibrate prevented the radiation-induced increase in microglial activation and the decrease in newborn hippocampal neurogenesis in young adult mice [63]. It is important to investigate if, similar to PPARα and PPARγ, PPARδ can modulate radiation-induced brain injury. Given that PPARδ is the predominant subtype in the brain, it is possible that PPARδ agonists can mediate a more pronounced anti-inflammatory response following irradiation [124]. Thus, we hypothesize that PPARδ activation will prevent/ameliorate radiation-induced brain injury, in part, by modulating inflammatory processes in microglia.
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 CHAPTER II  
MATERIALS AND METHODS  

Materials for in vitro studies: The PPARδ agonist, L-165041, was purchased from Calbiochem (San Diego, CA). The MEK inhibitor, U0126, the ERK1/2 inhibitor, FR180204, and the PKCα/β inhibitor, G06976, were purchased from EMD millipore (La Jolla, CA), Santa-Cruz Biotechnologies (Santa-Cruz, CA), and EMD millipore, respectively. All drugs were dissolved in Me₂SO₄ (DMSO). Goat anti-Cox-2, rabbit anti-MEK1/2, rabbit anti-p-MEK1/2, mouse anti-ERK1/2, mouse-anti-p-ERK1/2, goat anti-p-c-jun, and mouse anti-p-IkBα were purchased from Santa-Cruz Biotechnologies. Rabbit anti-p65, rabbit anti-PKCα, and rabbit anti-MCP-1 were purchased from Cell Signaling (Danvers, MA). Rabbit anti-p-PKCα was purchased from Epitomics (Burlingame, CA), and mouse anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and irradiation: BV-2 cells, immortalized murine microglial cells, were cultured in DMEM high glucose media (Invitrogen, Carlsbad, CA) supplied with 5% fetal bovine serum (Sigma-Aldrich), 100 IU/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich) and were maintained at 37°C in 10% CO₂ and 90% air. Twenty-four hours prior to irradiation, the culture medium was replaced with serum-free media. Cells were irradiated with a single dose of 10 Gy using a ¹³⁷Cs irradiator (J.L. Shepherd and Associates, San Fernando, CA) at a dose rate of 3.56 Gy/min. Irradiations were conducted at room temperature and control cells received sham-irradiation. Following irradiation, culture dishes were returned to the incubator and maintained at 37°C in 10% CO₂ and 90% air.

Short hairpin RNA targeting: Short hairpin RNA (shRNA) were generated as described by Sui et al. [1]. The PPARδ target site is: GGACCAGAACACACGCTTCCTT. BV-2 cells were infected with ecotopic virus and infected cells were selected by puromycin selection (Sigma-
Aldrich). Single-cell clones were generated, and the expression of PPARδ was evaluated by western blot analysis.

**Overexpression:** BV-2 cells were transiently transfected with the pcDNA3.1.PPARδ (Invitrogen) or the pcDNA3.1 empty vector control using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

**Luciferase assay:** Cells were plated on 24-well plates; 24 h later, cells were cotransfected with i) 0.2 μg of PPRE-ACOX (PPRE consensus sequence for the rat acyl-CoA oxidase gene, a kind gift from Dr. Thomas McIntyre, Univ. of Utah) or pGL3 control vector (Promega, Madison, WI), and ii) 0.02 μg pRL-SV40 renilla vector (a kind gift from Dr. Lee Yong Woo, Univ. of Virginia). Lipofectamine 2000 (Invitrogen) was used to transfect cells according to the manufacturer’s protocol. Transfected cells were treated with vehicle control or L-165041. Twenty-four hours later, the Dual Luciferase Assay (Promega) was conducted according to the manufacturer’s protocol, and the Terner Designs Reporter Microplate Luminometer (Promega) was used to measure luciferase activity. Fold change in luminescence, a measure of luciferase activity, was calculated by the relative luminescence units (RLU) of firefly/RLU renilla luciferase.

**Measurement of intracellular ROS generation:** Intracellular ROS generation was measured using 2’7’-dichlorofluorescein (DCFH-DA) as previously described [2]. DCFH-DA is permeable to cell membranes; once in cells, it is cleaved by cellular esterases and becomes impermeable. When oxidized by ROS, the probe becomes fluorescent. Briefly, cells were washed with PBS+ (1x PBS supplemented with 0.14 g/L CaCl2 and 0.1 g/L of MgCl2), incubated with 10 μM DCFH-DA (Invitrogen, CA/Molecular Probes, Eugene, OR) for 45 min, washed with PBS+ to remove excess probe, and then incubated with 5 μM L-165041. Three hours after L-165041 treatment, cells were irradiated with a single dose of 10 Gy of $^{137}$Cs γ rays. One hour post-irradiation, ROS
generation was measured using a FACS BD Calibur (Becton Dickinson, Bedford, MA), and BD CellQuest™ Pro 6.0 software was used to analyze the data.

**RNA isolation and qRT-PCR:** RNA was harvested using Trizol reagent (Invitrogen) 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 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 45 cycles of 95°C for 15 min, 55°C for 30 sec, and 72°C for 30 sec. The fold changes in MCP-1, IL-1β, and TNF-α gene expression were calculated using the comparative C_t (cross threshold) method. In brief, the C_t of the housekeeping gene GAPDH was subtracted from the C_t of MCP-1, IL-1β, or TNF-α to get ΔC_t. The ΔC_t of the sham-irradiated group was then subtracted from the ΔC_t for each of the other treatment groups to get ΔΔC_t. Fold changes compared to the sham-irradiated group were determined by calculating 2^-ΔΔC_t. Data represent the mean ± S.E.M of three independent experiments.

**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 Na_3VO_4 (Sigma-Aldrich), and 150 mM of NaCl. Lysates were centrifuged at 12,500 rpm for 10 min, and the supernatant was collected. Protein concentrations were measured using the Bradford assay (BioRad, Hercules, CA) at absorbance 595 nm. Five to 50 μg of protein were separated by SDS-PAGE. Protein was transferred to polyvinylidene fluoride (PVDF) membrane 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 primary antibody. 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 was conducted to quantify the signal intensity using Adobe Photoshop Elements 6.0.

**Electromobility shift assay (EMSA):** Cells were lysed on ice with Buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT); lysates were homogenized using a dounce homogenizer, which was followed by centrifugation at 12,000 rpm for 10 min. To extract nuclear protein, the nuclear pellets were lysed with Buffer C (5 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 25% v/v glycerol, 400 mM NaCl, 1 mM EDTA 0.5 mM DTT, 0.5 mM PMSF, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 1 mM Na₃VO₄). This was followed by centrifugation at 12,000 rpm for 10 min. Protein concentrations were measured using the Bradford assay (BioRad) at absorbance 595 nm. The EMSA procedure was performed using the Promega Gel-Shift Core Assay following the manufacturer's protocol. In brief, 10 μg of nuclear protein were incubated with 2 μL Binding Buffer (Promega) for 10 min. Consensus binding sequences of NF-κB (5'-AGTTGAGGGACTTCCCCAGGC-3' and 3'TCAACTCCCTGAAAGGTCGGC-5') and AP-1 (5'-CGCTTGATGAGTCAGCCGAA-3' and 3'-GCGAACTACTCAGTCGGCCTT-5') were labeled with 10 μCi γ-P32 (GE Healthcare, Piscataway, NJ) and T4 polynucleotide kinase (Promega). The consensus sequences were then incubated with the nuclear protein for 20 min and electrophoresed on a 4% non-denaturing polyacrylamide gel. An X-ray film was then placed on top of the gel and developed overnight at -80°C. The X-ray film was processed using a Kodak Processing System. Films were scanned and densitometry was performed.

**Co-immunoprecipitation:** Cell lysates were collected using RIPA buffer (50mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1% SDS, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 10 mg/mL PMSF, and 1 mM Na₃VO₄). Lysates were incubated with 2 μg anti-p65 at 4°C for 3 h, and immunocomplexes were captured by incubating lysates with Protein A/G PLUS agarose beads (Santa Cruz) at 4°C overnight. Agarose beads were centrifuged and washed 8
times with RIPA buffer. After centrifugation, the pellet was diluted with 80 μl 4x SDS-PAGE sample buffer (0.25M Tris-HCL, pH 6.8, 8% SDS, 30% Glycerol, 0.02% Bromophenol Blue, and 10% B-ME), and boiled for 5 min. The samples were then subjected to electrophoresis, and immunoblotted with an anti-PPARδ antibody.

**Statistical analysis of in vitro studies:** Each experiment was repeated a minimum of three independent times. All analyses were carried out using SAS software (SAS Inc, Cary, NC). Although the sample size within each treatment group is not large, we believe that the outcome measure distribution is normally distributed in the population. To determine statistical significance between two treatment groups, when appropriate, either a one- (when compared to sham) or two-sample t test was used. If more than two treatment groups were compared, analysis of variance (ANOVA) was used to explore the overall association. Bonferroni and Tukey’s studentized range tests were used for pairwise comparisons. Levene’s test was used to examine the homogeneity of variance, an assumption of variance. When this assumption was not valid, the Kruskal-Wallis test was used.

**Animals and irradiation procedures:** Adult (12-16 weeks old) C57Bl/6 wild-type (WT) mice (Jackson Laboratories, Bar Harbor, Maine) and PPARδ knockout (KO) mice (NIH, Bethesda, Maryland) were housed in a specific pathogen free environment, five mice per cage with free access to drinking water and standard mouse chow (Harlan Teklad, Madison, WI). 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 four experimental groups (n=3-8 per group), including: 1) sham-irradiation and control diet, 2) sham-irradiation and GW0742 (100ppm, GlaxoSmithKline, Research Triangle Park, NC), 3) WBI and control diet, and 4) WBI and GW0742. The composition of the control diet has been described previously [3]. Mice were placed on their respective diets 14 days prior to WBI and maintained on their diets until
euthanized (1-week or 2-months post-WBI) or for 3-months post-WBI (mice tested on the Barnes Maze task). Mice were anesthetized using a ketamine/xylazine mixture injected i.p. (90/10 mg/kg body weight [BW]) prior to irradiation. WBI was performed, as previously described, using a 7,214 Ci self-shielded $^{137}$Cs irradiator using lead and Cerrobend shielding devices [3]. Irradiated mice received a single dose of 10 Gy γ-rays at a dose rate of ~4 Gy/min with half the dose (5 Gy) delivered to each side of the head. Sham-irradiated mice were anesthetized but were not irradiated.

**Bromodeoxyuridine (BrdU) injection:** One month post-WBI, mice received an i.p. injection of 50 mg/kg BW of BrdU every day for 7 days. Two months post-WBI (three weeks following the last BrdU injection), mice were euthanized and tissues were processed as described below.

**Tissue processing:** Mice were anesthetized using a ketamine/xylazine mixture (200/10 mg/kg BW, i.p.). For CD68 and NeuN/BrdU staining, brains were isolated, 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-µm 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 protein and RNA.

**Immunohistochemistry and immunofluorescence:** A 1-in-6 (CD68 staining) or a 1-in-12 (NeuN/BrdU) series of sections from the entire 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$_2$O$_2$ in 1X TBS to block endogenous peroxidase activity and incubated overnight at 4°C with the rat α-CD68 (labels activated microglia; 1:100; AbD Serotec, Raleigh, NC), or rabbit α-GFAP (labels astrocytes; 1:7000; Dako, Carpinteria, CA) primary antibody. Staining was
detected using a biotinylated secondary antibody (1:200, Vectorlabs, Burlingame, CA) and visualized using peroxidase-conjugated avidin-biotin complex (ABC Elite kit) with nickel-enhanced DAB substrate (Vectorlabs, Burlingame, CA). 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 incubated overnight at 4°C with rat α-BrdU (1:200; AbD Serotec, Raleigh, NC) and mouse α-NeuN (1:200; Chemicon, Billerica, MA). 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), the DNA binding fluorescent dye, was used to visualize anatomical landmarks of the DG.

Quantitative analysis: Stereologic quantification is not biased by the shape, size, or orientation of the cells counted or by volumetric variation and, thus, is considered superior to traditional, single-section estimation techniques [4]. All analyses were performed blinded on coded sections. CD68 immunolabeled activated microglia or GFAP immunolabeled astrocytes were counted in the granule cell layer (GCL) and hilus of the DG in the hippocampus. CD68 or GFAP counts were normalized to the volume of the GCL and the hilus. All counts were performed on a Zeiss Imager D2 using StereoInvestigator software (Microbrightfield, Inc, Colchester, VT). The counting parameters for CD68+ or GFAP+ cells in the GCL/Hilus were as follows: sampling grid size (100 x 100 µm), counting frame size (75 x 75 µm), disector height (15 µm), and guard-zone thickness (2 µm). We determined the coefficient of error (CE) using the Gundersen-Jensen CE estimator estimates to estimate 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 that is, the ratio between CE² and observed variance of the group, CV², should be less than 0.5 [5, 6]. All estimates in the current study were less than 0.5.
The BrdU+ and BrdU+/NeuN+ positive cells were counted (in the GCL and subgranular zone [SGZ]) in stacks of optical sections acquired using a Leica TCS SP2 confocal microscope (Leica Microsystems, Bannockburn, IL). Counts were expressed as the number of NeuN+/BrdU+ in the GCL and SGZ.

**Barnes Maze task:** The Barnes Maze task is a sensitive task for detecting hippocampal-dependent spatial memory impairments following irradiation [7, 8]. The Barnes Maze contains a 121.9 cm in diameter white Plexiglas circular arena with 18 equidistant holes (5.5 cm in diameter, spaced every 20°, and centered 4.5 cm from the outer perimeter). Beneath one target hole was a black Plexiglas escape box (30 × 14 × 6 cm); the position of which was maintained for each mouse. A unique picture was positioned on each cardinal compass point to provide visual cues for the task. The Barnes Maze was illuminated brightly with 2 × 34 W diffuse overhead fluorescent lighting. 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 the container was lifted, mice had 1 min to explore the Maze. Mice were then guided to the escape box, a cover was placed over the hole to prevent escape from the escape box, and mice were habituated to the escape box for 1 min. This was repeated three times. On days 2-5 and 7-11, mice performed two 3 min trials as described above. 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 the escape box. On days 6 and 12, mice performed one probe trial (1 min duration) as described above except that the escape box was removed. 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 for subsequent analysis using automated video tracking system and software (Ethovision, Nodulus, Leesburg, VA, USA).

**Statistical Analysis of in vivo studies:** For all immunohistochemical and molecular data, an analysis of variance (ANOVA) was used to determine statistical significance between more than two experimental groups. Tukey’s studentized range test was used for pairwise comparisons. When only two experimental groups were compared, 2-sample t-tests were used and Bonferroni correction was used to correct for multiple comparisons.

For all Barnes Maze data, the latency to tunnel and distance to tunnel over the course of training trials were analyzed using a generalized estimating equation (GEE) and a two-factor repeated-measures ANOVA (radiation x drug x trial) to determine the rate of acquisition of the spatial learning task. These analyses were followed by a pair-wise comparison, controlling for multiple comparisons, to determine which time points were significantly different from the sham group. In order to analyze probe data to determine if there was a radiation and/or drug effect, a two-way ANOVA was used (radiation x drug). In all cases, p< 0.05 was considered significant.
References


CHAPTER III

PPARδ prevents radiation-induced proinflammatory responses in microglia via transrepression of NF-κB and inhibition of the PKCα/MEK1/2/ERK1/2/AP-1 pathway

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Abstract

Partial or whole-brain irradiation is often required to treat both primary and metastatic brain cancer. Radiation-induced normal tissue injury, including progressive cognitive impairment, however, can significantly affect the well-being of the approximately 200,000 patients who receive these treatments each year in the US. Although the exact mechanisms underlying radiation-induced late effects remain unclear, oxidative stress and inflammation are thought to play a critical role. Microglia are key mediators of neuroinflammation. Peroxisomal proliferator-activated receptor (PPAR)δ has been shown to be a potent regulator of anti-inflammatory responses. Thus, we hypothesized that PPARδ activation would modulate the radiation-induced inflammatory response in microglia. Incubating BV-2 murine microglial cells with the PPARδ agonist, L-165041, prevented the radiation-induced increase in: i) intracellular ROS generation, ii) Cox-2 and MCP-1 expression, and iii) IL-1β and TNF-α message levels. This occurred, in part, through PPARδ-mediated modulation of stress activated kinases and proinflammatory transcription factors. PPARδ inhibited NF-κB via transrepression by physically interacting with the p65 subunit, and prevented activation of the PKCα/MEK1/2/ERK1/2/AP-1 pathway by inhibiting the radiation-induced increase in intracellular ROS generation. These data support the hypothesis that PPARδ activation can modulate radiation-induced oxidative stress and inflammatory responses in microglia.

Keywords
Ionizing radiation; PPARδ; radiation-induced brain injury; microglia; inflammation; NF-κB; PKCα/MEK1/2/ERK1/2/AP-1 pathway
Each year, approximately 200,000 patients will receive fractionated partial or whole-brain irradiation (fWBI) as treatment for primary or metastatic brain cancer [1]. Unfortunately, the radiation dose that can be delivered to the tumor is limited by the risk of toxicity to the surrounding normal brain tissue. Radiation-induced brain injury can lead to cognitive deficits several months to years after irradiation that affect patients’ quality of life (QOL) [2-4]. This diminished QOL has become an important concern for these long-term survivors of brain irradiation, and is recognized as one of the most important measurements of brain tumor therapy outcomes in clinical trials, second only to survival [5, 6]. Although clinical trials have demonstrated that short-term interventions can modulate cognitive impairment [7], there are no proven long-term treatments for radiation-induced cognitive deficits; therefore, it is important to investigate new therapeutic approaches [8].

Although it was once believed that the brain was not susceptible to inflammation, studies have now demonstrated that inflammatory responses do occur and may contribute to radiation-induced brain injury. *In vivo* studies indicate that there is an increase in proinflammatory mediators within hours of irradiating the rodent brain [9-11]. Microglia are considered to be one of the key mediators of neuroinflammation [12-15]. In an uninjured brain, ramified microglia actively monitor the microenvironment to ensure that the brain is maintaining homeostasis [12]. Following injury, microglia become activated, a process characterized by rounding of the cell body, retraction of cell processes, proliferation, and an increased production of cytokines, chemokines, and reactive oxygen species (ROS) [12-15]. Although microglial activation plays an important role in phagocytosis of dead cells, sustained activation is thought to contribute to a chronic proinflammatory state in the brain [13, 15]. *In vivo* studies in rodents indicate that radiation leads to an increase in microglial activation [16, 17]. Irradiating microglia cells *in vitro* leads to an increase in proinflammatory mediators, such as the cytokines TNF-α and IL-1β, and the chemokines MCP-1 and ICAM-1 [18-20].
Radiation-induced chronic oxidative stress and inflammatory responses produced by microglia may: i) lead to a decrease in neurogenesis in the hippocampus, a critical region for learning and memory; and/or ii) alter the environment of the neurogenic niche and, in turn, the functions of the pre-existing neurons [15, 16, 21]. Studies in rodents demonstrate that the administration of anti-inflammatory drugs can decrease radiation-induced microglial activation. This decrease has been associated with an improvement in hippocampal neurogenesis [16, 17]. Moreover, administration of eicosapentaenoic acid, a polyunsaturated fatty acid with anti-inflammatory properties, restored the altered long-term potentiation (LTP) of hippocampal slices following irradiation of the rat brain [21]. LTP is thought to underlie memory and cognitive function; it is a measure of signal transmission between two neurons [22]. These findings provide a strong rationale for investigating anti-inflammatory therapies to mitigate radiation-induced brain injury.

PPARδ is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors and one of three subtypes (α, δ, and γ) that make up the PPAR family [23, 24]. PPARs regulate transcription by heterodimerizing with the retinoid X receptor (RXR) and binding to PPAR response elements (PPREs). PPREs, located in the enhancer regions of genes, consist of an AGGTCA hexameric direct repeat separated by one or two nucleotides [25]. PPAR subtypes are encoded by different genes, have unique tissue distributions, and exhibit overlapping and differential functions. PPARδ is thought to be expressed ubiquitously; it is the predominant PPAR subtype in the CNS [26]. Several studies have demonstrated that PPARδ activation can modulate oxidative stress and inflammatory processes [27-29]. PPARδ mediates many of its anti-inflammatory effects by preventing activation of stress-activated kinases and proinflammatory transcription factors [30, 31]. In addition, PPARδ has been shown to regulate oxidative stress by activating transcription of antioxidant genes, such as catalase and superoxide dismutase [32, 33].
Recent studies suggest that PPARδ agonists may ameliorate the severity of various acute and chronic CNS pathologies, including stroke, multiple sclerosis, and Alzheimer's disease, in large part, by modulating the oxidative stress and proinflammatory responses associated with these diseases [34-36]. The role of PPARδ in the modulation of radiation-induced brain injury is unknown. We hypothesized that activation of PPARδ would inhibit the radiation-induced oxidative stress and proinflammatory responses in microglia. Here, we report that PPARδ activation does indeed prevent the radiation-induced increase in: i) intracellular ROS generation, ii) Cox-2 and MCP-1 protein, and iii) IL-1β and TNF-α message levels. This occurred, in part, by transrepression of NF-κB and inhibition of the PKCα/MEK1/2/ERK1/2/AP-1 pathway.
Materials and methods

Materials

The PPARδ agonist, L-165041, was purchased from Calbiochem (San Diego, CA). The MEK inhibitor, U0126, the ERK1/2 inhibitor, FR180204, and the PKCα/β inhibitor, G06976, were purchased from EMD millipore (La Jolla, CA), Santa-Cruz Biotechnologies (Santa-Cruz, CA), and EMD millipore, respectively. All drugs were dissolved in Me$_2$SO (DMSO). Goat anti-Cox-2, rabbit anti-MEK1/2, rabbit anti-p-MEK1/2, mouse anti-ERK1/2, mouse anti-p-ERK1/2, goat anti-p-c-jun, and mouse anti-p-IkBα were purchased from Santa-Cruz Biotechnologies. Rabbit anti-p65, rabbit anti-PKCα, and rabbit anti-MCP-1 were purchased from Cell Signaling (Danvers, MA). Rabbit anti-p-PKCα was purchased from Epitomics (Burlingame, CA), and mouse anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and Irradiation

BV-2 cells, immortalized murine microglial cells, were cultured in DMEM high glucose media (Invitrogen, Carlsbad, CA) supplied with 5% fetal bovine serum (Sigma-Aldrich), 100 IU/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich) and were maintained at 37°C in 10% CO$_2$ and 90% air. Twenty-four hours prior to irradiation, the culture medium was replaced with serum-free media. Cells were irradiated with a single dose of 10 Gy using a $^{137}$Cs irradiator (J.L. Shepherd and Associates, San Fernando, CA) at a dose rate of 3.56 Gy/min. Irradiations were conducted at room temperature and control cells received sham-irradiation. Following irradiation, culture dishes were returned to the incubator and maintained at 37°C in 10% CO$_2$ and 90% air.

Short hairpin RNA targeting

Short hairpin RNA (shRNA) were generated as described by Sui et al. [37]. The PPARδ target site is: GGACCAGAACACACGCTTCCTT. BV-2 cells were infected with ecotropic virus
and infected cells were selected by puromycin selection (Sigma-Aldrich). Single-cell clones were generated, and the expression of PPARδ was evaluated by western blot analysis.

**Overexpression**

BV-2 cells were transfected with the pcDNA3.1.PPARδ (Invitrogen) or the pcDNA3.1 empty vector control using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

**Luciferase assay**

Cells were plated on 24-well plates; 24 h later, cells were cotransfected with i) 0.2 μg of PPRE-ACOX (PPRE consensus sequence for the rat acyl-CoA oxidase gene, a kind gift from Dr. Thomas McIntyre, Univ. of Utah) or pGL3 control vector (Promega, Madison, WI), and ii) 0.02 μg pRL-SV40 renilla vector (a kind gift from Dr. Lee Yong Woo, Univ. of Virginia). Lipofectamine 2000 (Invitrogen) was used to transfect cells according to the manufacturer’s protocol. Transfected cells were treated with vehicle control or L-165041. Twenty-four hours later, the Dual Luciferase Assay (Promega) was conducted according to the manufacturer’s protocol, and the Terner Designs Reporter Microplate Luminometer (Promega) was used to measure luciferase activity. Fold change in luminescence, a measure of luciferase activity, was calculated by the relative luminescence units (RLU) of firefly/RLU renilla luciferase.

**Measurement of intracellular ROS generation**

Intracellular ROS generation was measured using 2’7’-dichlorofluorescein (DCFH-DA) as previously described [38]. DCFH-DA is permeable to cell membranes; once in cells, it is cleaved by cellular esterases and becomes impermeable. When oxidized by ROS, the probe becomes fluorescent. Briefly, cells were washed with PBS+ (1x PBS supplemented with 0.14 g/L CaCl2 and 0.1 g/L of MgCl2), incubated with 10 μM DCFH-DA (Invitrogen, CA/Molecular
Probes, Eugene, OR) for 45 min, washed with PBS+ to remove excess probe, and then incubated with 5 μM L-165041. Three hours after L-165041 treatment, cells were irradiated with a single dose of 10 Gy of $^{137}$Cs γ rays. One hour post-irradiation, ROS generation was measured using a FACS BDCalibur (Becton Dickinson, Bedford, MA), and BD CellQuest™ Pro 6.0 software was used to analyze the data.

**RNA isolation and qRT-PCR Syber Green**

RNA was harvested using Trizol reagent (Invitrogen) 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 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 45 cycles of 95°C for 15 min, 55°C for 30 sec, and 72°C for 30 sec. The fold changes in MCP-1, IL-1β, and TNF-α gene expression were calculated using the comparative C\text{t} (cross threshold) method. In brief, the C\text{t} of the housekeeping gene GAPDH was subtracted from the C\text{t} of MCP-1, IL-1β, or TNF-α to get ΔC\text{t}. The ΔC\text{t} of the sham-irradiated group was then subtracted from the ΔC\text{t} for each of the other treatment groups to get ΔΔC\text{t}. Fold changes compared to the sham-irradiated group were determined by calculating $2^{-ΔΔC\text{t}}$. Data represent the mean ± S.E.M of three independent experiments.

**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 Na$_3$VO$_4$ (Sigma-Aldrich), and 150 mM of NaCl. Lysates were centrifuged at 12,500 rpm for 10 min, and the
supernatant was collected. Protein concentrations were measured using the Bradford assay (BioRad, Hercules, CA) at absorbance 595 nm. Five to 50 μg of protein were separated by SDS-PAGE. Protein was transferred to polyvinylidene fluoride (PVDF) membrane 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 primary antibody. 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 was conducted to quantify the signal intensity using Adobe Photoshop Elements 6.0.

**Electromobility shift assay (EMSA)**

Cells were lysed on ice with Buffer A (10 mM Heps, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT); lysates were homogenized using a dounce homogenizer, which was followed by centrifugation at 12,000 rpm for 10 min. To extract nuclear protein, the nuclear pellets were lysed with Buffer C (5 mM Heps, pH 7.9, 1.5 mM MgCl₂, 25% v/v glycerol, 400 mM NaCl, 1 mM EDTA 0.5 mM DTT, 0.5 mM PMSF, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 1 mM Na₃VO₄). This was followed by centrifugation at 12,000 rpm for 10 min. Protein concentrations were measured using the Bradford assay (BioRad) at absorbance 595 nm. The EMSA procedure was performed using the Promega Gel-Shift Core Assay following the manufacturer's protocol. In brief, 10 μg of nuclear protein were incubated with 2 μL Binding Buffer (Promega) for 10 min. Consensus binding sequences of NF-κB (5’-AGTTGAGGGGACTTTCCCAGGC-3’ and 3’TCAACTCCCCTGAAAGGGTCCG-5’) and AP-1 (5’-CGCTTGATGAGTCAGCCGGAA-3’ and 3’-GCGAACTACTCAGTGGGCTTT-5’) were labeled with 10 μCi γ-P32 (GE Healthcare, Piscataway, NJ) and T4 polynucleotide kinase (Promega). The consensus sequences were then incubated with the nuclear protein for 20 min and electrophoresed on a 4% non-denaturing polyacrylamide gel. An X-ray film was then placed on top of the gel and developed overnight at
-80°C. The X-ray film was processed using a Kodak Processing System. Films were scanned and densitometry was performed.

Co-Immunoprecipitation

Cell lysates were collected using RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1% SDS, 1 mg/mL aprotinin, 1 mg/mL leupetin, 10 mg/mL PMSF, and 1 mM Na₃VO₄). Lysates were incubated with 2 μg anti-p65 at 4°C for 3 h, and immunocomplexes were captured by incubating lysates with Protein A/G PLUS agarose beads (Santa Cruz) at 4°C overnight. Agarose beads were centrifuged and washed 8 times with RIPA buffer. After centrifugation, the pellet was diluted with 80 μl 4x SDS-PAGE sample buffer (0.25 M Tris-HCL, pH 6.8, 8% SDS, 30% Glycerol, 0.02% Bromophenol Blue, and 10% B-ME), and boiled for 5 min. The samples were then subjected to electrophoresis, and immunoblotted with an anti-PPARδ antibody.

Statistical Analysis

Each experiment was repeated a minimum of three independent times. All analyses were carried out using SAS software (SAS Inc, Cary, NC). Although the sample size within each treatment group is not large, we believe that the outcome measure distribution is normally distributed in the population. To determine statistical significance between two treatment groups, when appropriate, either a one- (when compared to sham) or two-sample t test was used. If more than two treatment groups were compared, analysis of variance (ANOVA) was used to explore the overall association. Bonferroni and Tukey’s studentized range tests were used for pairwise comparisons. Levene’s test was used to examine the homogeneity of variance, an assumption of variance. When this assumption was not valid, the Kruskal-Wallis test was used.
Results

**BV-2 cells express a functional PPARδ**

To demonstrate that BV-2 cells contain a functional PPARδ and were appropriate for our studies, we performed a luciferase reporter assay. Cells were co-transfected with: i) a PPRE-driven luciferase vector or a pGL3 control vector, and ii) a renilla vector. They were then incubated with the PPARδ agonist, L-165041, or vehicle control for 24 h, and luciferase activity was measured. Incubating BV-2 cells with 5 μM of L-165041 led to a 2-fold increase in luciferase activity, suggesting that these cells do express a functional PPARδ (SFig. 1). Since 5 μM of L-165041 led to a significant increase in luciferase fluorescence, we chose to use this concentration of the PPARδ agonist for the remainder of our studies.

**PPARδ activation prevents the radiation-induced increase in intracellular ROS generation and proinflammatory mediators in BV-2 cells**

Radiation induces increased intracellular ROS generation in BV-2 cells [18]; we hypothesized that PPARδ activation could modulate this response. To test this hypothesis, we used the oxidation sensitive probe DCFH-DA to measure intracellular ROS generation in BV-2 cells, which were incubated with L-165041 prior to irradiation with a single dose of 10 Gy. As predicted, irradiating the cells resulted in increased ROS production 1 h post-irradiation, and this response was inhibited in cells treated with L-165041 (Fig. 1A). Incubating the cells with n-acetyl-cysteine, an ROS scavenger, also inhibited the radiation-induced increase in DCF fluorescence (Fig. 1A). As expected, the non-oxidizable control probe, carboxy-DCF (C369) did not show any radiation-induced difference in fluorescence (Fig. 1A).

Previous reports have demonstrated that irradiating BV-2 cells leads to an increase in Cox-2 protein and IL-1β and TNF-α message levels [18, 19]. We hypothesized that PPARδ activation would inhibit the radiation-induced inflammatory response in BV-2 cells. As shown in Fig. 1B (SFig. 2), L-165041 inhibited the increase in Cox-2 and MCP-1 protein levels observed 7
h post-irradiation. The increase in IL-1β and TNF-α message levels determined 24 h post-irradiation was also significantly inhibited when cells were pretreated with L-165041 (Fig. 1C).

This effect appeared to be PPARδ-dependent; L-165041 failed to inhibit the radiation-induced increase in Cox-2 in the presence of the PPARδ antagonist GSK0660 (SFig. 3) [39]. Moreover, overexpressing PPARδ by transfecting cells with the pcDNA3.1.PPARδ vector inhibited the radiation-induced increase in Cox-2 expression (Fig. 2A). In contrast, shRNA-mediated knockdown of PPARδ (Fig. 2B) led to an increase in the radiation-induced Cox-2 and MCP-1 expression compared to cells infected with scrambled control shRNA. These results indicate that: i] overexpression of PPARδ prevents the radiation-induced increase in Cox-2 expression, and ii] loss of PPARδ enhances the radiation-induced increase in Cox-2 and MCP-1 expression. We also examined if PPARδ knockdown leads to an increase in IL-1β or TNF-α message levels, or intracellular ROS generation; however, we did not observe any differences in these endpoints between cells expressing scramble- or PPARδ-targeted shRNA (data not shown).

**PPARδ activation inhibits NF-κB activation via transrepression by physically interacting with the p65 subunit**

The radiation-induced proinflammatory response in BV-2 cells is regulated, in part, by NF-κB [18, 19]. Thus, we examined if PPARδ activation could modulate NF-κB activation in BV-2 cells following irradiation. As predicted, pre-incubating BV-2 cells with L-165041 prevented the radiation-induced increase in NF-κB DNA binding observed 30 min post-irradiation (Fig 3A; SFig 4A). To investigate the mechanism by which PPARδ modulates NF-κB activation, we examined the phosphorylation of Iκ-Bα and the nuclear translocation of p65. Thirty minutes post-irradiation, we observed the predicted increases in the phosphorylation of Iκ-Bα at Ser 32 and the nuclear translocation of p65; L-165041 did not modulate these responses (Figs. 3B & 3C; SFig 4B and SFig 4C). Next, we examined if PPARδ inhibited NF-κB activation via
transrepression by binding to the p65 subunit. Indeed, when we immunoprecipitated cellular lysates for p65 and then immunoblotted for PPARδ, we observed that L-165041 led to an increase in the binding of PPARδ to p65 30 min post-irradiation (Fig. 3D). Mechanistically, these data suggest that PPARδ activation prevents the DNA binding activity of NF-κB via transrepression by physically interacting with the p65 subunit.

*PPARδ activation prevents AP-1 activation by inhibiting c-jun phosphorylation and upstream activation of the stress-activated kinases MEK1/2 and ERK1/2*

In addition to NF-κB activation, activation of AP-1 is thought to underlie the radiation-induced increase in proinflammatory mediators in BV-2 cells [18]. We therefore investigated if L-165041 could also modulate AP-1 activation. Pre-incubating BV-2 cells with L-165041 prevented the radiation-induced increase in AP-1 DNA binding activity observed 30 min post-irradiation (Fig. 4A; SFig. 5A). Activation of AP-1, a dimeric protein, is mediated, in part, by phosphorylation of the c-jun subunit. We observed that L-165041 inhibited the phosphorylation of nuclear c-jun at Ser73 following irradiation, suggesting that PPARδ activation inhibits AP-1 activation by preventing c-jun activation (Fig. 4B; SFig. 5B).

MEK1/2 and ERK1/2 activation regulates c-jun phosphorylation and AP-1 activation in BV-2 cells [Zhiyong Deng and Weiling Zhao, unpublished data]. Interestingly, studies have demonstrated that PPARδ can modulate activation of the MEK1/2/ERK1/2 pathway [31, 40]. Thus, we hypothesized that PPARδ modulates AP-1 activation via inhibition of MEK1/2 and ERK1/2 phosphorylation. Consistent with our hypothesis, pre-incubating BV-2 cells with L-165041 reduced the radiation-induced phosphorylation of MEK1/2 at Ser218 and Ser222, and that of ERK1/2 at Thr 202 and Tyr 204 (Figs. 4C & 4D; SFig. 5C & 5D). Furthermore, pretreating BV-2 cells with 2 μM of the MEK1/2 inhibitor, U0126, or 2 μM of the ERK1/2 inhibitor, FR180204, inhibited the radiation-induced increase in intracellular ROS generation and
Cox-2 expression (Figs. 5A & 5B; SFigs. 6). MEK1/2 or ERK1/2 inhibition also prevented the radiation-induced increases in MCP-1, IL-1β, and TNF-α gene expression (Figs. 6A & 6B).

**PPARδ modulates MEK1/2 and ERK1/2 activation, in part, by inhibiting the radiation-induced phosphorylation and expression of PKCα**

PKCα is an activator of the MEK1/2/ERK1/2 pathway [41, 42]. A radiation-induced activation of PKCα has not been previously reported in microglia. However, lipopolysaccaride (LPS), an inflammatory stimulus, has been shown to increase activation of PKCα in microglial cells [43]. Therefore, we next investigated if radiation induces PKCα activation in BV-2 cells, and further, if PPARδ activation can modulate this response. As shown in Fig. 7A (SFig. 7A), irradiating BV-2 cells with a single dose of 10 Gy led to an increase in the phosphorylation of PKCα at Thr638 and the expression of PKCα, and pre-treatment with L-165041 prevented these increases.

To demonstrate that inhibition of PKCα prevents the radiation-induced activation of MEK1/2 and ERK1/2, we pre-treated BV-2 cells with 1 μM of the PKCα/β inhibitor, Go6976, and examined the phosphorylation of MEK1/2 and ERK1/2 30 min post-irradiation. As predicted, Go6976 prevented the radiation-induced phosphorylation of MEK1/2 and ERK1/2 (Figs. 7B & 7C; SFigs. 7B & 7C).

The radiation-induced phosphorylation of MEK1/2 and ERK1/2 is modulated by ROS production in BV-2 cells [Zhiyong Deng and Weiling Zhao, unpublished data]. Our results suggest that PKCα regulates MEK1/2 and ERK1/2 phosphorylation; thus, we examined whether PKCα phosphorylation is also modulated by ROS production. Indeed, pre-treating BV-2 cells with 10 mM of NAC inhibited the radiation-induced phosphorylation and expression of PKCα (Fig. 7D; SFig. 7D). Given that L-165041 inhibited the radiation-induced increase in intracellular ROS generation (Fig. 1A), these data suggest that PPARδ activation negatively regulates the PKCα/MEK1/2/ERK1/2 pathway by preventing ROS generation following irradiation.
Discussion

A growing body of evidence suggests that PPARδ activation can regulate oxidative stress and inflammatory responses following various cellular stresses. We therefore hypothesized that PPARδ activation would prevent the radiation-induced oxidative stress and proinflammatory responses in microglia. As predicted, pretreating BV-2 cells with L-165041 inhibited the radiation-induced increase in: i) intracellular ROS generation, ii) Cox-2 and MCP-1 protein expression, and iii) IL-1β and TNF-α message levels. This occurred, in part, by negatively regulating the DNA binding of NF-κB and AP-1. PPARδ activation: i) inhibited NF-κB via transrepression by physically interacting with the p65 subunit, and ii) prevented activation of the PKCα/MEK1/2/ERK1/2/AP-1 pathway by inhibiting the radiation-induced increase in intracellular ROS generation.

The radiation-induced proinflammatory response in BV-2 cells is regulated, in part, by NF-κB. There are several mechanisms by which PPARδ can modulate NF-κB, including: i) inhibiting the nuclear translocation of p65, ii) binding directly to p65, and iii) reducing acetylation of p65 [30, 44, 45]. In a rat model of reperfusion injury, the PPARδ agonist, GW0742, reduced myocardial infarct size, in part, by inhibiting nuclear translocation of p65 [44]. In cultured neonatal rat cardiomyocytes, L-165041 decreased LPS-stimulated NF-κB activation by increasing the physical interaction of PPARδ with the p65 subunit. This interaction is thought to interfere with the DNA binding of NF-κB [30]. The PPARδ agonist, GW501516, inhibited inflammation in human HaCaT keratinocytes by reducing TNF-α-induced p65 acetylation. Acetylation of p65 is regulated by the transcriptional co-activator p300; reduced acetylation of p65 decreases DNA binding and activation of NF-κB [45]. Our studies indicate that L-165041 increased the physical interaction of PPARδ to p65, suggesting that the receptor modulates NF-κB activation via transrepression.

In addition to NF-κB activation, the radiation-induced proinflammatory response in BV-2 cells is regulated, in part, by AP-1 activation. The transactivation function of the c-jun subunit of
AP-1 depends on the phosphorylation of residues Ser63 and Ser73 [46]. Radiation induces phosphorylation of both residues in BV-2 cells [18]. We observed that PPARδ prevents the phosphorylation of nuclear c-jun at Ser73, and further, inhibits AP-1 DNA binding. Previous studies in BV-2 cells demonstrate that c-jun is phosphorylated by ERK1/2 [Zhiyong Deng and Weiling Zhao, unpublished data]. We observed that PPARδ activation prevents the radiation-induced activation of MEK1/2 and ERK1/2. In addition to AP-1, activation of the MEK1/2/ERK1/2 pathway has been shown to modulate NF-κB activation. In white adipose tissue, PPARδ activation prevented LPS-induced inflammation by inhibiting ERK1/2 activation and the downstream activation of NF-κB [40]. It is therefore possible that the PPARδ-mediated modulation of the MEK1/2/ERK1/2 pathway in our model also contributes to a reduction in radiation-induced proinflammatory responses, in part, by preventing NF-κB activation.

PKC is an upstream regulator of the MEK1/2/ERK1/2 pathway. In BV-2 cells, LPS-stimulated PKC activation led to ERK1/2 phosphorylation and increased Cox-2 protein levels [47]. Furthermore, in cultures of primary microglia, LPS-stimulated PKCα modulated TNF-α expression [43]. Studies have demonstrated that PPARδ can modulate PKCα. In PPARδ KO mice compared to wild-type mice, TPA induced significantly greater PKCα activity, enhancing MEK1/2 and ERK1/2 phosphorylation and Cox-2 expression [31]. Moreover, studies in platelets have demonstrated that PPARδ can physically interact with PKCα and suppress PKCα-mediated platelet activation [48]. Given the evidence that PKCα modulates both the MEK1/2/ERK1/2 pathway and inflammation, and further that PPARδ modulates PKCα activity, we examined if PKCα was an activator of MEK1/2 and ERK1/2 in our model. Indeed, we observed that the radiation-induced phosphorylation of PKCα activates the MEK1/2/ERK1/2 pathway, and PPARδ activation inhibits this response.

Our data suggest that PPARδ prevented activation of the PKCα/MEK1/2/ERK1/2/AP-1 pathway by inhibiting the radiation-induced increase in intracellular ROS generation. Of interest, MEK1/2 and ERK1/2 inhibition also prevented the radiation-induced increase in intracellular
ROS generation, suggesting that the increase in intracellular ROS generation observed following irradiation reflects both direct and indirect effects, and that PPARδ activation can inhibit these responses. The antioxidant actions of PPARδ agonists have been observed in a variety of cell types [27-29]; we observed that PPARδ activation could inhibit intracellular ROS generation following irradiation of BV-2 cells. It should be noted that PPARδ can activate transcription of several antioxidant genes, including catalase, superoxide dismutase, glutathione peroxidase 1, heme oxygenase 1, and thioredoxin 1 [32, 33]. Previous studies in our lab have failed to detect changes in the expression levels of antioxidant genes in BV-2 cells; however, changes in the activity levels of antioxidant enzymes have not been examined [18]. Future studies should examine if radiation induces changes in the activity levels of antioxidant enzymes, and further, if PPARδ can modulate these changes. These studies will help elucidate the mechanisms by which PPARδ activation can modulate intracellular ROS.

Based on the findings described above, we propose a model, outlined in Fig. 8, for the role of PPARδ in modulating radiation-induced proinflammatory responses in microglia. Following irradiation, increased intracellular ROS generation leads to activation of the stress-activated kinases, PKCα, MEK1/2, and ERK1/2, and the proinflammatory transcription factors, NF-κB and AP-1. Activation of these transcription factors increases the expression of Cox-2, MCP-1, IL-1β, and TNF-α; these proinflammatory mediators contribute to the inflammatory phenotype of microglia. PPARδ activation prevents the radiation-induced proinflammatory response, in part, by negatively regulating NF-κB and AP-1. Specifically, PPARδ physically interacts with p65 and inhibits the DNA binding of NF-κB. Furthermore, PPARδ inhibits the radiation-induced increase in intracellular ROS generation, which prevents PKCα, MEK1/2, ERK1/2, and c-jun phosphorylation and the DNA binding of AP-1. Taken together, our data indicate that PPARδ activation can prevent the acute radiation-induced oxidative stress and proinflammatory responses observed in microglia within hours of in vitro irradiation.
It is important to note that the microglia used in our in vitro studies were grown under 21% oxygen, a concentration much higher than would occur in vivo. Thus, the affect of “physiological” oxygen concentration on the radiation-induced inflammatory response of microglial cells remains to be determined. Furthermore, although our in vitro studies examined the radiation-induced inflammatory response in microglial cells, radiation-induced brain injury in vivo is a multicellular process. Indeed, we recognize that interpreting these data in terms of their relevance to the onset and progression of radiation-induced late effects in the brain is difficult and somewhat controversial. It is unlikely that the radiation-induced cognitive impairment observed 6 months or more after fWBI is a direct result of acute proinflammatory responses in the microglia alone. However, brain irradiation clearly leads to chronic, persistent increases in activated microglia [16, 17]; the resultant neuroinflammation has been associated with decreased neurogenesis and impaired neuronal function [15, 16, 21]. Thus, understanding the mechanisms by which radiation alters the microglia cell phenotype, and how PPARδ agonists might prevent/ameliorate these changes, offers the promise of identifying potential interventions that can be evaluated in vivo.

Moreover, since PPARδ activation has been shown to regulate inflammation in multiple cell types and promote the survival of neurons under stress conditions, it is possible that PPARδ can mitigate radiation-induced brain injury in multiple cell types [29, 49]. Additionally, animal studies have demonstrated that PPARδ agonists can cross the blood brain barrier and modulate oxidative stress and proinflammatory responses associated with acute and chronic CNS disorders [34-36]. Overall, our current in vitro findings indicate that PPARδ activation shows promise as a potential therapeutic strategy in the treatment and/or prevention of radiation-induced brain injury.
Acknowledgements

This work was supported by NIH grant CA112593 (MER). We thank Dr. Linda van Eldik, Northwestern University, USA, for generously providing the BV-2 cells, originally developed by Dr. V. Bocchini, University of Perugia, Italy. We thank Dr. Weiling Zhao (Department of Radiation Oncology, WFSM) for providing antibodies against phosphorylated c-jun and p65.
FIGURE LEGENDS

Figure 1. PPARδ activation prevents the radiation-induced increases in intracellular ROS generation, Cox-2 and MCP-1 protein, and IL-1β and TNF-α mRNA levels. A, BV-2 cells were incubated with 10 μM DCFH-DA or 10 μM C369 for 45 min, the probe was washed off using PBS+, and the cells were pretreated with 5 μM L-165041, NAC, or vehicle control for 3 h. The cells were then irradiated with a single dose of 10 Gy of 137Cs γ rays or sham irradiated, and intracellular ROS generation was measured 1 h post-irradiation as described in the Materials and methods. The results are presented as arbitrary fluorescence units. B & C, BV-2 cells were pretreated with 5 μM of L-165401 and irradiated with a single dose of 10 Gy. B, Protein was harvested 7 h post-irradiation and subjected to western blot analysis for Cox-2 or MCP-1; β-actin was used as a loading control. See SFig. 2 for densitometric analysis. C, RNA was harvested 24 h post-irradiation; the expression levels of IL-1β and TNF-α were analyzed using SYBER Green Real-time PCR and normalized with GAPDH expression levels. Changes in gene expression were calculated using the 2^(-ΔΔCt) methods (see Materials and methods). Mean ± S.E.M; *, p≤ 0.05 vs. sham-irradiated, #, p≤ 0.05 vs. 10 Gy; n=3.
Figure 2. Overexpression or knockdown of PPARδ modulates Cox-2 and MCP-1 expression in BV-2 cells. A, BV-2 cells were transfected with 24 μg of the pcDNA3.1PPARδ vector or 24 μg of the empty pcDNA3.1 vector using Lipofectamine 2000 according to the manufacturer’s protocol. Twenty-four h post-transfection, cells were serum starved for 24 h, treated for 3 h with 5 μM L-165041 or vehicle control, irradiated with a single dose of 10 Gy, and protein was harvested 7 h post-irradiation. Protein was subjected to western blot analysis for Cox-2; β-actin was used as a loading control. B, BV-2 cells were infected with scramble control or shRNA targeting PPARδ. Single knockdown clones were selected, irradiated with a single dose of 10 Gy, and protein was harvested 7 h post-irradiation. Protein was subjected to western blot analysis for Cox-2 or MCP-1. β-actin was used as a loading control.
Figure 3. PPARδ activation prevents radiation-induced NF-κB activation by physical interaction with the p65 subunit. BV-2 cells were pretreated with 5 μM of L-165401 or vehicle control and irradiated with a single dose of 10 Gy. A, Nuclear protein was collected 30 min post-irradiation. Gel-Shift analysis was carried out by incubating 10 μg of nuclear protein with γ-ATP P\textsuperscript{32} end-labeled NF-κB consensus oligo (see Materials and methods). The samples were run on a 4% non-denaturing acrylamide gel, stained with 7% Acetic acid, and exposed to X-ray film. B, Protein was harvested 30 min post-irradiation; whole cell lysates were subjected to western blot analysis for p-IκBα. C, Nuclear protein was harvested 30 min post-irradiation; nuclear protein was subjected to western blot analysis for nuclear p65. B & C, β-actin was used as a loading control. A-C, See SFig. 4 for densitometric analysis. D, Co-immunoprecipitation was carried out using anti-p65 and immunoblotting for PPARδ (see Materials and methods).
Figure 4. PPARδ activation prevents radiation-induced AP-1 activation and upstream activation of c-jun, MEK1/2, and ERK1/2. BV-2 cells were pretreated with 5 μM of L-165041 or vehicle control and irradiated with a single dose of 10 Gy. A, Nuclear protein was collected 30 min post-irradiation. Gel-Shift analysis was carried out by incubating 10 μg of nuclear protein with γ-ATP P<sup>32</sup> end-labeled AP-1 consensus oligo (see Materials and methods). The samples were run on a 4% non-denaturing acrylamide gel, stained with 7% Acetic acid, and exposed to X-ray film. B, Nuclear protein was collected 30 min post-irradiation; nuclear protein was subjected to western blot analysis for p-c-jun; β-actin was used as a loading control. C & D, Protein was harvested 30 min post-irradiation and subjected to western blot analysis for C, p-MEK1/2 and total-MEK1/2 and D, p-ERK1/2 and total-ERK1/2; β-actin was used as a loading control. A-D, See SFig. 5 for densitometric analysis.
Figure 5. The MEK1/2 inhibitor, U0126, or the ERK1/2 inhibitor, FR180204, prevent the radiation-induced increase in intracellular ROS generation and Cox-2 expression. A, BV-2 cells were incubated with 10 μM DCFH-DA for 45 min, the probe was washed off using PBS+, and the cells were pretreated with 2 μM U0126, 2 μM FR180204, or vehicle control for 3 h. The cells were then irradiated with a single dose of 10 Gy of $^{137}$Cs γ rays or sham irradiated, and intracellular ROS generation was measured 1 h post-irradiation. B, BV-2 cells were pretreated with 2 μM U0126 or 2 μM FR180204, or vehicle control for 1 h prior to treatment with 5 μM L-165041 or vehicle control. Three hours post-L-165041 treatment, cells were irradiated with a single dose of 10 Gy and whole cell lysates were collected. Protein was subjected to western blot analysis for Cox-2. β-actin was used as a loading control. See SFig. 6 for densitometric analysis.
Figure 6. MEK1/2 or ERK1/2 inhibition prevents the radiation-induced increase in MCP-1, IL-1β, and TNF-α gene expression. BV-2 cells were pretreated with A, 2 μM U0126, B, 2 μM FR180204, or vehicle control for 3 h. Three h post-treatment, cells were irradiated with a single dose of 10 Gy and RNA was harvested 24 h post-irradiation. The expression levels of MCP-1, IL-1β, and TNF-α were analyzed using SYBER Green Real-time PCR and normalized with GAPDH expression levels. Changes in gene expression were calculated using the 2^ΔΔCt methods (see Materials and methods). Mean ± S.E.M; *, p≤ 0.05 vs. sham-irradiated, #, p≤ 0.05 vs. 10 Gy; n=3.
Figure 7. PPARδ modulates MEK1/2 and ERK1/2 activation, in part, by inhibiting upstream activation of PKCα. BV-2 cells were pretreated with A, 5 μM L-165041, B & C, 1 μM Go6976, D, 10 mM NAC, or vehicle control 3 h prior to a single dose of 10 Gy. Protein was harvested 30 min post-irradiation and subjected to western blot analysis for A & D, p-PKCα and total-PKCα, B, p-MEK1/2 and total-MEK1/2, or C, p-ERK1/2 and total-ERK1/2. A-D, β-actin was used as a loading control. A-D, See SFig. 7 for densitometric analysis.
Figure 8. Proposed model outlining the role of PPARδ in the modulation of the radiation-induced proinflammatory response in BV-2 cells. Irradiation of BV-2 cells leads to an increase in intracellular ROS generation. This increases activation of the stress-activated kinases, PKCα, MEK1/2, and ERK1/2, and the proinflammatory transcription factors, NF-κB and AP-1. Activation of NF-κB and AP-1 enhance the expression of Cox-2, MCP-1, IL-1β, and TNF-α. PPARδ activation prevents radiation-induced neuroinflammation, in part, by transrepression of NF-κB by physical interaction with the p65 subunit. Additionally, PPARδ inhibits intracellular ROS generation, which prevents PKCα, MEK, and ERK1/2 phosphorylation and downstream activation of AP-1.
Figure S1. A functional PPARδ is expressed in BV-2 cells. BV-2 cells were cotransfected using Lipofectamine 2000 (Invitrogen, Carlsbad, California) with i) 0.2 μg of PPRE-ACOX (PPRE consensus rat acyl-CoA oxidase gene) or pGL3 (control vector) and ii) 0.02 μg of pRL-SV40 (a renilla plasmid). Twenty-four h post-transfection, cells were treated with 0, 1, or 5 μM of L-165041 or vehicle control. The Dual Luciferase Assay (Promega, Madison, Wisconsin) was used to measure luciferase activity according to the manufacturer’s instructions. * = p≤ 0.05.

Figure S2. PPARδ activation prevents the radiation-induced increases in Cox-2 and MCP-1 protein expression. Densitometric analysis of Fig. 1B. Results are shown as fold changes compared to sham-irradiated controls. Mean ± S.E.M; *, p≤0.05 vs. sham; #, p≤0.05 vs. 10 Gy; n = 3.
Figure S3. The PPARδ antagonist, GSK0660, prevents the L-165041-mediated inhibition of the radiation-induced increase in Cox-2 protein. BV-2 cells were treated with 10 μM of the PPARδ antagonist GSK0660 or vehicle control 1 h prior to treatment with 5 μM L-165041 or vehicle control. Cells were then irradiated with a single dose of 10 Gy and protein was subjected to western blot analysis for Cox-2; β-actin was used as a loading control.

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[Western Blot Image: Cox-2 and β-actin]
Figure S4. PPARδ activation prevents radiation-induced NF-κB activation. Densitometric analysis of Figs 3A-3C (A-C). Results are shown as fold changes compared to sham-irradiated controls.

A.

Fold Change NF-κB Binding

B.

Fold Change kBp1-β-actin

C.

Fold Change nuclear p65/β-actin
Figure S5. PPARδ activation prevents radiation-induced AP-1 activation and upstream activation of c-jun, MEK1/2, and ERK1/2. Densitometric analysis of Figs 4A-4D (A-D). Results are shown as fold changes compared to sham-irradiated controls. Mean ± S.E.M; *, p≤0.05 vs. sham; #, p≤0.05 vs. 10 Gy; n = 3.
Figure S6. The MEK1/2 inhibitor, U0126, or the ERK1/2 inhibitor, FR180204, prevent the radiation-induced increase in Cox-2 expression. Densitometric analysis of Figs 5B. Results are shown as fold changes compared to sham-irradiated cells. Mean ± S.E.M; *, p≤0.05 vs. sham; n = 3.
Figure S7. PPARδ modulates MEK1/2/ERK1/2 activation, in part, by inhibiting upstream activation of PKCα. Densitometric analysis of Figs 7A-7D (A-D). Results are shown in fold changes compared to sham-irradiated controls. Mean ± S.E.M; *, p≤0.05 vs. sham; #, p≤0.05 vs. 10 Gy; n = 3.
References


CHAPTER IV

The PPARδ agonist, GW0742, inhibits microglial activation and inflammation but does not restore neurogenesis after mouse whole-brain irradiation

Caroline I. Schnegg, Dana Greene-Schloesser, Mitra Kooshki, Valerie S. Payne, Fang-Chi Hsu, Mike E. Robbins

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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 contributes, in part, to the pathogenesis of radiation-induced brain injury. In this study, we investigated whether administration of the peroxisomal proliferator-activated receptor (PPAR)δ agonist, [4-[[2-[3-Fluoro-4-(trifluoromethyl)phenyl]-4-methyl-5-thiazolyl[methyl[thio]-2-methylphenoxy]acetic acid (GW0742), prevented radiation-induced brain injury in C57Bl/6 wild-type (WT) mice. Our data demonstrate that GW0742 prevented the radiation-induced increase in the number of activated microglia (CD68+ cells) in WT mice 1 week following 10 Gy WBI. Furthermore, GW0742 inhibited the WBI-induced increase in IL-1β message levels and ERK phosphorylation observed 3 h post-irradiation. In contrast, GW0742 administration failed to modulate the radiation-induced decrease in hippocampal neurogenesis (NeuN+/BrdU+ cells) determined 2 months after irradiation, or mitigate hippocampal-dependent spatial memory impairment observed 3 months post-irradiation using the Barnes Maze task. We used PPARδ knockout (KO) mice to examine if the effects of GW0742 were PPARδ-dependent. Unexpectedly, PPARδ KO mice exhibited a differential response following WBI compared to WT mice; therefore, we were unable to make mechanistic conclusions about GW0742. Interestingly, the number of GFAP+ cells was reduced significantly in the WT mice 2 months after WBI, but this was not observed in the PPARδ KO mice. These results demonstrate that: i) GW0742 prevents the radiation-induced increase in microglial activation and inflammatory markers; ii) GW0742 does not restore altered hippocampal neurogenesis following WBI, or ameliorate early delayed hippocampal-dependent cognitive impairment; and iii) WT and PPARδ KO mice have a differential response to WBI.
Introduction

Partial or fractionated whole-brain irradiation (fWBI) often is required to treat both primary and metastatic brain cancer [1]. Radiation-induced normal tissue injury, including progressive cognitive impairment, however, can affect significantly the quality of life (QOL) of the patients who receive these treatments each year [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 measurements of brain tumor therapy outcomes in clinical trials [5]. Radiation-induced cognitive deficits often present as impairments in hippocampal-dependent learning and memory, as well as spatial information processing and executive function [6]. Clinical trials have demonstrated that short-term interventions can modulate cognitive deficits; however, there are no proven long-term treatments for radiation-induced cognitive impairment [7]. It is, therefore, important to investigate new therapeutic strategies to prevent/ameliorate radiation-induced brain injury [8].

Neuroinflammation is a key aspect of the brain’s response to radiation and is often characterized by an increase in the number of activated microglia [9, 10]. Radiation-induced chronic inflammatory responses produced by microglia modify the brain’s microenvironment and, in turn, alter many neural processes [11-13]. Studies in rodents indicate that there is a negative correlation between microglial activation and hippocampal neurogenesis following irradiation [11]. The WBI-induced decrease in neurogenesis is associated with defects in hippocampal-dependent learning and memory [6]. Anti-inflammatory drugs have been shown to inhibit the radiation-induced increase in microglial activation, and concomitantly, ameliorate/prevent the radiation-induced decrease in hippocampal neurogenesis in rodents [11, 14]. Thus, anti-inflammatory drugs may be a beneficial therapy to mitigate 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 [15, 16].
Furthermore, administration of PPARδ agonists confer neuroprotection following various acute and chronic injuries to the central nervous system (CNS), including Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, and stroke, in large part, by modulating inflammatory markers associated with the diseases [17-19]. We previously reported that pre-treating BV-2 murine microglial cells with the PPARδ agonist, L-165041, prevented the radiation-induced increase in inflammatory markers by negative regulation of the NF-κB and PKCα/MEK/ERK/AP-1 pathways [20]. Data from our lab also demonstrate that L-165041 pretreatment inhibits the radiation-induced increase in IL-6 and MCP-1 message levels, as well as the increase in GFAP message and protein levels, in part, by preventing the activation of the PKCα/MEK1/2/ERK1/2 pathway (Robbins unpublished observations). In this study, we hypothesized that administration of the PPARδ agonist, GW0742, in the mouse diet would mitigate WBI-induced brain injury, including cognitive impairment.
Materials and Methods

Animals and irradiation procedures

Adult (12-16 weeks old) C57Bl/6 wild-type (WT) mice (Jackson Laboratories, Bar Harbor, Maine) and PPARδ knockout (KO) mice (NIH, Bethesda, Maryland) were housed in a specific pathogen free environment, five mice per cage with free access to drinking water and standard mouse chow (Harlan Teklad, Madison, WI). 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 four experimental groups (n=3-8 per group), including: 1) sham-irradiation and control diet, 2) sham-irradiation and GW0742 (100ppm, GlaxoSmithKline, Research Triangle Park, NC), 3) WBI and control diet, and 4) WBI and GW0742. The composition of the control diet has been described previously [14]. Mice were placed on their respective diets 14 days prior to WBI and maintained on their diets until euthanized (1-week or 2-months post-WBI) or for 3-months post-WBI (mice tested on the Barnes maze task). Mice were anesthetized using a ketamine/xylazine mixture injected i.p. (90/10 mg/kg body weight [BW]) prior to irradiation. WBI was performed, as previously described, using a 7,214 Ci self-shielded 137Cs irradiator using lead and Cerrobend shielding devices [14]. Irradiated mice received a single dose of 10 Gy γ-rays at a dose rate of ~4 Gy/min with half the dose (5 Gy) delivered to each side of the head. Sham-irradiated mice were anesthetized but were not irradiated.

Bromodeoxyuridine (BrdU) injection

One month post-WBI, mice received an i.p. injection of 50 mg/kg BW of BrdU every day for 7 days. Two months post-WBI (three weeks following the last BrdU injection), mice were euthanized and tissues were processed as described below.
**Tissue processing**

Mice were anesthetized using a ketamine/xylose mixture (200/10 mg/kg BW, i.p.). For CD68 and NeuN/BrdU staining, brains were isolated, 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-µm 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 protein and RNA.

**Immunohistochemistry and immunofluorescence**

A 1-in-6 (CD68 staining) or a 1-in-12 (NeuN/BrdU) series of sections from the entire 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<sub>2</sub>O<sub>2</sub> in 1X TBS to block endogenous peroxidase activity and incubated overnight at 4°C with the rat α-CD68 (labels activated microglia; 1:100; AbD Serotec, Raleigh, NC), or rabbit α-GFAP (labels astrocytes; 1:7000; Dako, Carpinteria, CA) primary antibody. Staining was detected using a biotinylated secondary antibody (1:200, Vectorlabs, Burlingame, CA) and visualized using peroxidase-conjugated avidin-biotin complex (ABC Elite kit) with nickel-enhanced DAB substrate (Vectorlabs, Burlingame, CA). 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 incubated overnight at 4°C with rat α-BrdU (1:200; AbD Serotec, Raleigh, NC) and mouse α-NeuN (1:200; Chemicon, Billerica, MA). 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), the DNA binding fluorescent dye, was used to visualize anatomical landmarks of the DG.

**Quantitative analysis**

Stereologic quantification is not biased by the shape, size, or orientation of the cells counted or by volumetric variation and, thus, is considered superior to traditional, single-section estimation techniques [21]. All analyses were performed blinded on coded sections. CD68 immunolabeled activated microglia or GFAP immunolabeled astrocytes were counted in the granule cell layer (GCL) and hilus of the DG in the hippocampus. All counts were performed on a Zeiss Imager D2 using StereoInvestigator software (Microbrightfield, Inc, Colchester, VT). The counting parameters for CD68⁺ or GFAP⁺ cells in the GCL/Hilus were as follows: sampling grid size (100 x 100 µm), counting frame size (75 x 75 µm), disector height (15 µm), and guard-zone thickness (2 µm). We determined the coefficient of error (CE) using the Gundersen-Jensen CE estimator estimates to estimate 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 that is, the ratio between CE² and observed variance of the group, CV², should be less than 0.5 [22, 23]. All estimates in the current study were less than 0.5.

The BrdU⁺ and BrdU⁺/NeuN⁺ positive cells were counted (in the GCL and subgranular zone [SGZ]) in stacks of optical sections acquired using a Leica TCS SP2 confocal microscope (Leica Microsystems, Bannockburn, IL). Counts were expressed as the number of NeuN+/BrdU+ in the GCL and SGZ.

**RNA isolation and qRT-PCR Syber Green**

RNA was harvested using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. DNA contamination was removed by acid-phenol chloroform extraction
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 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 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 Ct (cross threshold) method. GAPDH was used as the loading control. Data represent the mean ± S.E.M of three individual mice.

**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 leupetin (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 was collected. Protein concentrations were measured using the Bradford assay (BioRad, Hercules, CA) at absorbance 595 nm. Protein was separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane 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 was conducted to quantify the signal intensity using Adobe Photoshop Elements 6.0.

**Barnes Maze Task**
The Barnes maze task is a sensitive task for detecting hippocampal-dependent spatial memory impairments following irradiation [24, 25]. The Barnes maze contains a 121.9 cm in diameter white Plexiglas circular arena with 18 equidistant holes (5.5 cm in diameter, spaced every 20°, and centered 4.5 cm from the outer perimeter). Beneath one target hole was a black Plexiglas escape box (30 × 14 × 6 cm); the position of which was maintained for each mouse. A unique picture was positioned on each cardinal compass point to provide visual cues for the task. The Barnes maze was illuminated brightly with 2 × 34 W diffuse overhead fluorescent lighting. 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 the container was lifted, mice had 1 min to explore the maze. Mice were then guided to the escape box, a cover was placed over the hole to prevent escape from the escape box, and mice were habituated to the escape box for 1 min. This was repeated three times. On days 2-5 and 7-11, mice performed two 3 min trials as described above. 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 the escape box. On days 6 and 12, mice performed one probe trial (1 min duration) as described above except that the escape box was removed. 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 for subsequent analysis using automated video tracking system and software (Ethovision, Nodulus, Leesburg, VA, USA).

**Statistical Analysis**

For all immunohistochemical and molecular data, an analysis of variance (ANOVA) was used to determine statistical significance between more than two experimental groups. Tukey’s
studentized range test was used for pairwise comparisons. When only two experimental groups were compared, 2-sample t-tests were used and Bonferroni correction was used to correct for multiple comparisons.

For all Barnes Maze data, the latency to tunnel and distance to tunnel over the course of training trials were analyzed using a generalized estimating equation (GEE) and a two-factor repeated-measures ANOVA (radiation x drug x trial) to determine the rate of acquisition of the spatial learning task. These analyses were followed by a pair-wise comparison, controlling for multiple comparisons, to determine which time points were significantly different from the sham group. In order to analyze probe data to determine if there was a radiation and/or drug effect, a two-way ANOVA was used (radiation x drug). In all cases, \( p < 0.05 \) was considered significant.

**Results**

*GW0742 administration prevented the WBI-induced increase in activated microglia in WT mice*

Neuroinflammation is hypothesized to contribute to radiation-induced cognitive impairment [11]. Studies from our lab indicate that PPARδ activation significantly inhibits the *in vitro* radiation-induced increase in proinflammatory mediators in microglia [20]. The PPARδ agonist, GW0742, has been shown to cross the blood brain barrier and ameliorate a variety of CNS disorders [18, 26, 27]. We hypothesized that GW0742 administration would prevent the radiation-induced increase in activated microglia. One week following a single dose of 10 Gy of \(^{137}\)Cs \( \gamma \) WBI, WT mice exhibited a significant increase in the number of activated microglia (CD68\(^+\) cells) in the dentate gyrus (DG) (Fig.1A). As predicted, this was prevented in irradiated mice that received GW0742 (Fig. 1A). Two months following WBI, the number of activated microglia was unaltered in WT mice (Fig. 1B). Thus, GW0742 administration prevented the increased microglial activation observed 1 week after WBI.
PPARδ KO mice have a differential response to WBI compared to WT mice

Studies have demonstrated that some of the effects of PPARδ agonists are independent of the receptor [28]. To address this concern, we determined if GW0742 administration prevented the increase in the number of activated microglia in PPARδ KO mice. Interestingly, the number of activated microglia was unaltered both 1 week and 2 months after WBI in the DG of the KO mice (Fig. 2A & 2B). Based on these findings, we were unable to make mechanistic conclusions regarding whether GW0742 is dependent on PPARδ. These results, however, suggest that PPARδ deficiency leads to an inhibition of microglial activation. These findings are surprising and quite different from those observed in PPARα KO mice, where PPARα deficiency resulted in a sustained increase in activated microglia observed at 1 week and 2 months post-WBI, and suggest that the effect of PPAR deficiency is subtype-dependent [14].

In addition to displaying a different microglial response following WBI, PPARδ KO mice also exhibited an altered astrocytic response. Whereas WBI in the WT mice was associated with a marked reduction in the number of astrocytes (GFAP+ cells) determined 2 months post-irradiation, there was no apparent decrease in the number of astrocytes in the irradiated PPARδ KO mice (Fig. 3).

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

We have reported previously that PPARδ activation prevents the radiation-induced increase in IL-1β message levels and ERK phosphorylation in microglia cells in vitro [20]; therefore, we examined if GW0742 administration inhibited these markers of inflammation in vivo observed 3 h post-WBI. As shown in Fig. 3A, WT mice exhibited an increase in IL-1β message levels 3 h after WBI. Administration of GW0742 prevented the radiation-induced increase in IL-1β message levels (Fig. 3A). We also observed an increase in ERK phosphorylation 3 h post-
irradiation in WT mice that was inhibited in the irradiated mice that received GW0742 (Fig. 3B). These results support the hypothesis that GW0742 administration prevents the acute radiation-induced increase in inflammatory markers in vivo.

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

Given that GW0742 prevented the radiation-induced increase in microglial activation and that brain inflammation has been shown to be detrimental to neurogenesis, we hypothesized that GW0742 would prevent the deleterious effect of WBI on hippocampal neurogenesis. As shown in Fig. 4, WBI led to an approximately 90% reduction in the number of BrdU+/NeuN+ cells in the GCL/SGZ of WT and KO mice 2 months post-irradiation. This effect was not inhibited in the irradiated WT or KO mice that received GW0742 in their diet (Fig. 4). These findings indicate that GW0742 does not restore altered hippocampal neurogenesis after brain irradiation.

**GW0742 does not mitigate the WBI-induced impairment in hippocampal-dependent spatial memory**

Three months after exposure to 10 Gy WBI, we determined that hippocampal-dependent spatial memory was impaired in WT mice using the latency of the mice to locate the escape box as an end point (Z=-2.64, p = 0.0083) (Fig. 5A). However, there was no drug (Z=1.01, p=0.3143) or interaction (Z=-0.04, p=0.9663) effect. Mice did not exhibit a difference in distance traveled to the escape box, and we did not observe a radiation (Z=-0.83, p=0.4081), drug (Z=1.35, p=0.1768), or interaction (Z=-1.27, p=0.2058) effect. During the first probe trial, the mice did not exhibit a difference in their frequency to the escape box zone; we did not observe a radiation (F(1,56)=0.21, p=0.6470), drug (F(1,56)=2.36, p=0.1305), or interaction effect (F(1,56)=0.21, p=0.6470) (Fig. 5B). However, during the second probe trial, the frequency in the escape box zone was altered by WBI (F(1,56)=4.38, p=0.0409), but there was no drug (F(1,56)=1.13, p=0.2933)
or interaction effect \(F_{(1,56)}=0.30, p=0.5881\). It therefore appears that GW0742 administration does not protect against early delayed changes in cognition. Six months following 10 Gy WBI, we did not observe any radiation-induced deficits in hippocampal-dependent spatial memory using the: i) latency to the escape box (radiation, \(Z=-0.20\), \(p=0.8409\); drug, \(Z=-0.27\), \(p=0.7899\); interaction, \(Z=1.33, p=0.1836\)); ii) distance to the escape box (radiation, \(Z=0.57\), \(p=0.5666\); drug, \(Z=0.43\), \(p=0.6679\)); or iii) frequency to the escape box zone during the first probe trial (radiation \(F_{(1,50)}=0.12, p=0.7299\), drug \(F_{(1,50)}=0.00, p=0.9517\), or interaction effect \(F_{(1,56)}=0.00, p=0.9547\)) as end points (Fig. 5C & 5D). However, there was a significant interaction effect for the distance to the tunnel (\(Z=-9.56, p<0.0001\)) and a significant drug effect for the second 6 month probe trial \(F_{(1,53)}=9.40, p=0.0034\) with the drug groups having a lower frequency to the escape box as compared to the Sham and radiation only groups. There was no significant radiation \(F_{(1,53)}=0.46, p=0.5017\) or interaction effect \(F_{(1,53)}=0.39, p=0.5332\)) for frequency to the tunnel for the second 6 month probe trial (Fig. 5C & 5D). Thus, 6 months post-irradiation, we did not observe defects in hippocampal-dependent memory due to radiation, suggesting that the deficits observed 3 months post-WBI were an early delayed response that did not lead to progressive cognitive impairment at 6 months.

**Discussion**

The main findings of this study were: i) GW0742 prevents the WBI-induced increase in activated microglia in WT mice; ii) PPARδ KO mice do not exhibit a WBI-induced increase in activated microglia; iii) WBI decreases the number of astrocytes in WT but not PPARδ KO iv) GW0742 inhibits the increase in IL-1β message levels and ERK phosphorylation post-WBI; v) GW0742 does not restore altered hippocampal neurogenesis following WBI; and vi) GW0742 does not mitigate early delayed hippocampal-dependent cognitive impairment observed 3 months post-WBI.
Microglia have many functions in the brain, including supporting the growth of neurons [29]. In response to various stimuli, such as radiation, microglia become activated and undergo physiological changes, including the upregulation of the lysosomal antigen macrosialin (CD68) and the release of proinflammatory factors [30]. Neuroinflammation is associated with radiation-induced brain injury [11]. Thus, mitigating the increase in proinflammatory responses following irradiation may be a rationale pharmacologic approach to modulate radiation-induced brain injury. Given the potent anti-inflammatory effects of PPARδ activation in microglia and astrocytes in vitro, we hypothesized that GW0742 would prevent the WBI-induced increase in activated microglia [20]. As predicted, administration of GW0742 to WT mice prevented the increase in the number of CD68+ cells in the DG 1 week following irradiation. Consistent with previous studies in our lab in 129S1/SvImJ mice, the number of activated microglia returned to control levels by 2 months post-WBI [14]. Thus, in our model, microglial activation appears to be an early event following irradiation. In addition, GW0742 administration to WT mice prevented the radiation-induced increase in IL-1β message levels and ERK phosphorylation—two markers of inflammation—3 hours after WBI. To our knowledge, this is the first demonstration of a role for PPARδ in modulating increased microglial activation and inflammatory markers following WBI.

Data demonstrate that GW0742 can act independently of PPARδ [28]. We, therefore, used PPARδ KO mice to investigate whether PPARδ is required for the anti-inflammatory properties of GW0742. Surprisingly, in our model, the number of activated microglia was unaltered both 1 week and 2 months after WBI in the DG of the KO mice. Thus, we were unable to make mechanistic conclusions regarding GW0742. These studies highlight the differential response of WT and PPARδ KO mice to WBI. In addition to displaying a different microglial response after WBI, PPARδ KO mice also exhibited an altered astrocytic response. We demonstrated that although the number of GFAP+ cells was decreased significantly in the
WT mice 2 months after WBI, it was not in the KO mice. These findings suggest that PPARδ regulates the number of astrocytes following WBI.

Studies have demonstrated that a negative correlation exists between activated microglia and neurogenesis; thus, we hypothesized that GW0742 administration would prevent or ameliorate the WBI-induced decrease in hippocampal neurogenesis [11, 13]. Although we did observe a decrease in hippocampal neurogenesis 2 months following WBI, GW0742 treatment did not modulate this response. Our findings are in agreement with previous data, suggesting that a simple link between inhibiting increased microglial activation and restoring hippocampal neurogenesis following irradiation is unlikely to exist [36, 37]. The effects of neuroinflammation on neurogenesis and cognition are still largely unknown [36, 37]. In particular, administration of the angiotensin-converting enzyme (ACE) inhibitor, ramipril, inhibited the increase in microglial activation and ameliorated cognitive impairment following fractionated WBI (fWBI), but did not restore hippocampal neurogenesis [37]. Furthermore, treatment with the angiotensin II type I receptor antagonist (AT₁RA), L-158,809, before, during, and after fWBI prevented or ameliorated cognitive impairment; however, the antagonist did not modulate radiation-induced inflammation or restore neurogenesis [36, 38]. Taken together, although radiation-induced cognitive impairment is associated with increased microglial activation and decreased neurogenesis, cognitive defects can be mitigated without concomitantly modulating microglial activation or neurogenesis.

Clinically, radiation-induced brain injury is characterized by progressive cognitive impairment [2, 6]. One limitation of our studies is that the cognitive deficits we observed in WT mice 3 months following irradiation were no longer present by 6 months following irradiation, suggesting that the impairments were an early delayed response rather than progressive cognitive impairment. Of note, our lab also has been unsuccessful in detecting hippocampal-dependent cognitive dysfunction in young adult male rats administered 40 Gy fWBI [37]. Our lab has, however, established an experimental rat model of radiation-induced brain injury to
measure perirhinal cortex dependent cognitive function using the Novel Object Recognition task; we have observed cognitive impairment 6 months to 1 year after fWBI [37, 38, 39]. Although GW0742 does not protect against early delayed changes in cognition, it is possible that the mechanisms underlying early delayed cognitive impairment are different than those contributing to progressive cognitive impairment. Future studies, therefore, could examine if administration of GW0742 can ameliorate and/or prevent radiation-induced cognitive impairment in young adult male rats using the Novel Object Recognition task.

In summary, we have shown that GW0742 prevents the deleterious effect of WBI on microglial activation. GW0742 administration also prevented the WBI-induced increase in IL-1β message levels and ERK phosphorylation. However, administration of GW0742 does not restore altered hippocampal neurogenesis following WBI, or ameliorate early delayed hippocampal-dependent cognitive impairment.
Figure 1. GW0742 administration prevented the WBI-induced increase in activated microglia in WT mice. A, A significant increase in CD68+ cells (activated microglia) was observed 1 week post-WBI in the dentate gyrus of WT mice; this increase was prevented in the mice administered GW0742 (100 ppm). B, Two months post-WBI, the numbers of CD68+ cells in WT mice were similar in all four treatment groups. Data are presented as Mean ± SEM; n=3 mice/group and an average of 8 sections were stained using α-CD68 antibody (1:100) and counted/animal; * p<0.05 vs. Sham; # p<0.05 vs. 10 Gy.
Figure 2. **PPARδ KO mice do not exhibit a WBI-induced increase in activated microglia.** Both A, 1 week post-WBI and B, 2 months post-WBI, the numbers of CD68+ cells in the DG of KO mice were similar in all four treatment groups. Data are presented as Mean ± SEM; n=3 mice/group and an average of 8 sections were stained using α-CD68 antibody (1:100) and counted/animal.
Figure 3. **WBI decreases GFAP+ cells in WT but not in PPARδ KO mice.** A significant decrease in the number of GFAP+ cells was observed in the DG of WT mice 2 months post-WBI. Data are presented as Mean ± SEM; n=3 mice/group and an average of 8 sections were stained using α-GFAP antibody (1:7000) and counted/animal; * p<0.05 vs. Sham WT.
Figure 4. GW0742 administration inhibited the WBI-induced increase in IL-1β and p-ERK. A, A significant increase in IL-1β message levels was observed 3 h post-WBI in the brains of WT mice; this increase was prevented in the mice administered GW0742. B, A significant increase in the phosphorylation of ERK was observed 3 h post-WBI in the brains of WT mice; this increase was prevented in the mice administered GW0742. A & B, Data are presented as Mean ± SEM; n=3 mice/group; * p<0.05 vs. Sham; # p<0.05 vs. 10 Gy.
Figure 5. GW0742 does not prevent the WBI-induced decrease in hippocampal neurogenesis. A significant decrease in the number of newborn neurons (BrdU+/NeuN+) in the GCL/SGZ was observed 2 months post-WBI; this decrease was not prevented in the mice administered GW0742. Data are presented as Mean ± SEM; n=8 mice/group; an average of 5 sections/animal were double-labeled fluorescently using α-BrdU (proliferation) and α-NeuN (neuron) antibodies and labeled cells counted using a confocal microscope; * p<0.05 vs. Sham.
Figure 6. GW0742 does not mitigate the WBI-induced impairment in hippocampal-dependent spatial memory. A, Three months post-WBI, the latency of WT mice to the escape box was increased (Z=-2.64, p = 0.0083). B, The frequency of WT mice in the escape box zone was decreased on the second probe day 3 months post-WBI (F=4.38, p=0.0409). C, Six months following post-WBI, the latency to the escape box was similar in all four treatment groups. D, The frequency of the mice administered GW0742 in the escape box zone was decreased on the second probe trial. Data are presented as Mean ± SEM; n=13-15 mice/group; * p<0.05 vs. Sham.
References


[38] Robbins, M. E.; Payne, V.; Tommasi, E.; Diz, D. I.; Hsu, F. C.; Brown, W. R.; Wheeler, K. T.; Olson, J.; Zhao, W. The AT1 receptor antagonist, L-158,809, prevents or ameliorates

In 2012, approximately 1.6 million new cancer cases will be diagnosed in the United States alone, of which approximately 30% will develop brain metastases [1]. Fractionated partial or whole-brain irradiation (fWBI) is often required to treat both primary and metastatic brain cancer. However, radiation-induced normal tissue injury, including cognitive impairment, can affect significantly the quality of life (QOL) of the approximately 200,000 patients who receive these treatments each year [2-4]. This diminished QOL has become an important concern for these long-term survivors of brain irradiation, and is recognized as one of the most important measurements of brain tumor therapy outcomes in clinical trials, second only to survival [5, 6]. Radiation-induced cognitive impairment will occur in a significant proportion of these patients with the incidence and severity of symptoms increasing with increased survival time. With better systemic agents improving the life expectancy of patients with cancer, patients with metastatic brain disease are living longer. Consequently, they are at a higher risk for developing radiation-induced cognitive impairment. Presently, there are no proven long-term treatments for radiation-induced cognitive deficits. It is, therefore, important to investigate new therapeutic approaches [7, 8].

Neuroinflammation is a key aspect of the brain’s response to radiation and is often characterized by an increase in the number of activated microglia [9, 10]. Radiation-induced chronic inflammatory responses produced by microglia modify the brain’s microenvironment and, in turn, alter many neural processes, including neurogenesis and synaptic transmission [11, 9]. Studies in rodents demonstrate that the administration of the anti-inflammatory drugs, indomethacin or fenofibrate, inhibited the radiation-induced increase in microglial activation; this decrease was associated with an improvement in hippocampal neurogenesis [10, 12]. Moreover, administration of eicosapentaenoic acid, a polyunsaturated fatty acid with anti-
inflammatory properties, restored the altered long-term potentiation (LTP) of hippocampal slices following irradiation of the rat brain [13]. LTP is thought to underlie memory and cognitive function; it is a measure of signal transmission between two neurons [14]. These findings provide a strong rationale for investigating anti-inflammatory therapies to mitigate radiation-induced brain injury.

The anti-inflammatory and anti-oxidant properties of PPARδ agonists have been characterized in multiple cell types. Recent studies suggest that PPARδ agonists may ameliorate the severity of various acute and chronic CNS pathologies, including stroke, multiple sclerosis, and Alzheimer’s disease, in large part, by modulating the oxidative stress and proinflammatory responses associated with these diseases [15-18]. Given the potent anti-inflammatory properties of PPARδ ligands, we hypothesized that PPARδ activation would mitigate radiation-induced brain injury, in part, by modulating the radiation-induced increase in oxidative stress and inflammatory markers in microglia.

Consistent with our hypothesis, we demonstrated that pretreating BV-2 murine microglia cells with the PPARδ agonist, L-165041, inhibited the radiation-induced increase in: i) intracellular ROS generation, ii) Cox-2 and MCP-1 protein expression, and iii) IL-1β and TNF-α message levels (Chapter III). These data established a relationship between PPARδ activation and the inhibition of pro-inflammatory markers in microglial cells following irradiation. A significant increase in the gene expression of TNF-α, IL-1β, Cox-2, and MCP-1 has been observed acutely 4-24 h following irradiation of the rodent brain [19-21]. Increased TNF-α message levels also have been observed chronically 6 months following irradiation of the rodent brain [20]. Although the relationship between acute changes in proinflammatory mediators and late effects in vivo has not been elucidated, there is evidence that proinflammatory mediators can lead to impairments in learning and memory. In particular, elevated levels of proinflammatory mediators, including TNFα, IL-1β, MCP-1 and Cox-2, have been observed in several CNS disorders, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis,
and cerebral ischemia [22-28]. Moreover, the overexpression of TNFα, IL-1β, or Cox-2 led to impairments in learning and memory in rodents, and the overexpression of TNFα or IL-1β altered the synaptic plasticity in the hippocampus of rodents [29-33]. Our data highlight the pleiotrophic effects of PPARδ activation on inflammation, as PPARδ modulates multiple markers of inflammation in microglia that might be involved in the development and progression of radiation-induced brain injury.

The radiation-induced proinflammatory response in BV-2 cells is regulated, in part, by NF-κB and AP-1 activation; thus, we examined if PPARδ inhibited the radiation-induced increase in the DNA binding of NF-κB and AP-1 [34]. As predicted, PPARδ inhibited the radiation-induced increase in NF-κB and AP-1 activation in BV-2 cells (Chapter III). Our studies suggest that PPARδ activation inhibited NF-κB via transrepression by physically interacting with the p65 subunit; this interaction is thought to interfere with the DNA binding of NF-κB (Chapter III) [35]. Furthermore, our data demonstrate that PPARδ inhibited AP-1 DNA binding by preventing the radiation-induced phosphorylation of nuclear c-jun at Ser73 (Chapter III). Although the AP-1 and NF-κB pathways are important for the normal development of the brain, their disregulation has been documented in several neurological disorders, including radiation-induced brain injury [36-39]. In particular, irradiation of the rodent brain leads to a significant increase in the DNA binding activity of AP-1 and NF-κB within hours following WBI [38, 39]. Moreover, neuronal precursor cell apoptosis following irradiation is thought to occur, in part, via c-jun phosphorylation [40]. Taken together, these studies suggest that activation of AP-1 and NF-κB might play a role in radiation-induced brain injury, and further, that PPARδ can modulate activation of these proinflammatory transcription factors in microglia.

Previous studies demonstrated that the radiation-induced activation of AP-1 in BV-2 cells is regulated by activation of MEK1/2 and ERK1/2 [Zhiyong Deng and Weiling Zhao, In Press]. Thus, we examined whether PPARδ activation modulates MEK1/2 and ERK1/2 phosphorylation. Indeed, we observed that PPARδ activation prevented the radiation-induced
activation of MEK1/2 and ERK1/2 (Chapter III). In addition, PPARδ prevented the radiation-induced increase in PKCα, an upstream regulator of the MEK1/2/ERK1/2 pathway (Chapter III). These studies established a novel relationship between the radiation-induced activation of PKCα and the proinflammatory response in BV-2 cells.

Since the radiation-induced phosphorylation of MEK1/2 and ERK1/2 is modulated by ROS production in BV-2 cells [Zhiyong Deng and Weiling Zhao, In Press], and further, that PKCα regulates MEK and ERK activation following irradiation, we investigated whether PKCα phosphorylation also is modulated by ROS production. As predicted, pre-treating BV-2 cells with 10 mM of NAC, an ROS scavenger, inhibited the radiation-induced phosphorylation and expression of PKCα (Chapter III). Given that L-165041 also inhibited the radiation-induced increase in intracellular ROS generation, these data suggest that PPARδ activation negatively regulates the PKCα/MEK1/2/ERK1/2/AP-1 pathway by preventing increased intracellular ROS generation following irradiation (Chapter III).

In summary, our in vitro findings demonstrate that PPARδ activation prevents the radiation-induced increase in oxidative stress and proinflammatory markers in microglia. This occurred, in part, through PPARδ-mediated modulation of stress activated kinases and proinflammatory transcription factors. PPARδ inhibited NF-κB via transrepression by physically interacting with the p65 subunit, and prevented activation of the PKCα/MEK1/2/ERK1/2/AP-1 pathway by inhibiting the radiation-induced increase in intracellular ROS generation. These in vitro findings established that PPARδ activation is a potent and efficacious pharmacological approach to inhibit radiation-induced proinflammatory markers in microglia, and thus, provided a strong rationale for investigating if PPARδ modulates radiation-induced brain injury in vivo.

Microglial activation in vivo commonly is used as an indicator of inflammation; activated microglia can be measured by labeling them for the lysosomal antigen, macrospilin, also known as CD68 [41]. In wild-type (WT) mice, we observed that administering the PPARδ agonist, GW0742, prevented the increase in the number of CD68+ cells in the dentate gyrus (DG)
observed 1-week post-WBI (Chapter IV). Consistent with previous studies in our lab in 129S1/SvImJ mice, the number of activated microglia returned to control levels by 2 months post-WBI [10]. Thus, in our model, microglial activation appears to be an early event following irradiation.

Studies have demonstrated that some of the effects of PPARδ agonists are independent of the receptor [42]. Therefore, to address this concern, we also examined if GW0742 administration prevented the increase in the number of activated microglia in PPARδ knockout (KO) mice. Interestingly, radiation did not induce an increase in activated microglia in the DG of PPARδ KO mice determined 1 week or 2 months post-WBI (Chapter IV). Based on these findings, we were unable to make mechanistic conclusions regarding whether the effects of GW0742 are PPARδ-dependent; however, these results suggest that PPARδ deficiency leads to an inhibition of microglial activation. These findings are surprising and quite different from those observed in PPARα KO mice where PPARα deficiency resulted in a sustained increase in activated microglia seen at 1 week and 2 months post-WBI [10]. This indicates that the effect of PPAR deficiency is subtype-dependent.

Measuring the levels of proinflammatory mediators secreted by activated microglia or reactive astrocytes in vivo has been a challenge; however, 3 h following WBI we observed an increase in the message levels of IL-1β in the brains of WT mice. We also observed an increase in ERK phosphorylation 3 h post-WBI in the brains of WT mice. Administration of GW0742 prevented both the WBI-induced increase in IL-1β message levels and ERK phosphorylation (Chapter IV). These results further indicate that GW0742 administration can modulate inflammatory markers in vivo. Moreover, they demonstrate that PPARδ can inhibit similar inflammatory markers in BV-2 cells in vitro and in the mouse brain in vivo.

Studies have reported that inhibiting the WBI-induced increase in microglial activation using anti-inflammatory agents can restore or partially restore hippocampal neurogenesis [10, 12]. Since GW0742 prevented the increase in microglial activation following WBI, we
hypothesized that GW0742 administration in the mouse diet would preserve neurogenesis. As expected, irradiating the brains of both our WT and KO mice led to a decrease in the number of newborn neurons in the DG determined 2 months post-irradiation (Chapter IV). However, this effect was not inhibited in the irradiated mice that received GW0742 in their diet (Chapter IV). Based on these findings and previous studies, a simple link between preventing increased microglial activation and restoring hippocampal neurogenesis following irradiation is unlikely [45, 46]. The effects of neuroinflammation on neurogenesis and cognition still largely are unknown. In particular, administration of the angiotensin-converting enzyme (ACE) inhibitor, ramipril, ameliorated cognitive impairment and inhibited the increase in microglial activation following WBI, but it did not restore hippocampal neurogenesis [46]. Furthermore, treatment with the angiotensin II type I receptor antagonist (AT1RA), L-158,809, before, during, and after fWBI prevented or ameliorated cognitive impairment; however, the antagonist did not modulate radiation-induced inflammation or restore neurogenesis [45]. Taken together, although radiation-induced cognitive impairment is associated with increased microglial activation and decreased neurogenesis, cognitive defects can be mitigated without concomitantly modulating microglial activation or neurogenesis.

Clinically, radiation-induced brain injury is characterized by progressive cognitive impairment [40, 47]. Three months after exposure to 10 Gy WBI, we determined that hippocampal-dependent spatial memory was impaired in WT mice using the Barnes Maze task. However, GW0742 administration did not protect against early delayed changes in cognition. Six months following 10 Gy WBI, we did not observe defects in hippocampal-dependent spatial memory, suggesting that the deficits were an early delayed response rather than progressive cognitive impairment. Of note, our lab also has been unsuccessful in detecting hippocampal-dependent cognitive dysfunction in young adult male rats administered 40 Gy fWBI. Our lab has, however, developed an experimental rat model of radiation-induced brain injury to measure perirhinal cortex dependent cognitive function using the Novel Object Recognition (NOR) task.
In this model, we observe cognitive impairment 6 months to 1 year after fWBI [48, 49]. Although GW0742 does not protect against early delayed changes in cognition, it is possible that the mechanisms underlying early delayed cognitive impairment are different than those contributing to progressive cognitive impairment. Future studies, therefore, could examine if administration of GW0742 can ameliorate and/or prevent radiation-induced cognitive impairment in young adult male rats using the NOR task.

It is also important to note that age is a major factor that influences radiation-induced brain injury. Aging causes a significant reduction in the basal levels of immature neurons, and concurrently, increases microglial activation [50]. Interestingly, WBI decreased the number of newborn neurons in young rats (8 weeks old), but not in old rats (28 months old), suggesting that a decrease in hippocampal neurogenesis does not contribute significantly to radiation-induced cognitive impairment in old animals. Furthermore, microglial activation is increased markedly in old animals compared to young adults, indicating that the microenvironment of the brain is different in old animals compared to young animals [50]. Although GW0742 did not prevent the radiation-induced decrease in hippocampal neurogenesis, the PPARδ agonist did prevent the increase in activated microglia following irradiation. Therefore, GW0742 may be an efficacious agent to prevent radiation-induced brain injury in older individuals.

In this body of work, we demonstrated that PPARδ activation inhibits the radiation-induced increase in inflammatory markers in microglia in vitro and prevents the deleterious effect of WBI on microglial activation in vivo. It is important to note, however, that radiation-induced brain injury involves modulation of various autocrine, paracrine, and juxtacrine signaling between different cell types in the brain, including microglia, astrocytes, oligodendrocytes, neurons, and endothelial cells [51-55]. Each cell type exhibits a different response to radiation. In the discussion below, the radiation response of astrocytes, oligodendrocytes, neurons, and brain endothelial cells will be discussed. Evidence suggesting that the PPARδ-mediated
modulation of radiation-induced brain injury may be multi-faceted and not restricted to one cell type also is discussed below.

Astrocytes are the most abundant glial cell in the brain; they constitute 50% of the glial cell population and outnumber neurons 9:1. They have many functions in the brain, including supporting neuronal growth, neuronal transmission, and synaptic plasticity [56]. Additionally, signaling between astrocytes and endothelial cells contributes to both the generation and maintenance of the BBB [57]. Astrocytes also protect endothelial cells from oxidative injury [58]. Therefore, any changes in astrocyte function may, in turn, affect neuronal function and BBB maintenance. Astrocytes become reactive in response to a stimulus, such as brain trauma, ischemia, or infection, and both the morphology and function of astrocytes are altered. Reactive astrocytes exhibit a hypertrophic soma and hypertrophic processes, and display increased expression of GFAP, vimentin, and nestin [59, 60]. Functional markers of astrocyte reactivity include proinflammatory mediators such as IL-6, Cox-2, and MCP-1 [61-63]. Reactive astrocytes have been shown to exacerbate the severity of a variety of CNS disorders, including amyotrophic lateral sclerosis and Huntington’s disease [64, 65]. The role of astrocytes in the progression of radiation-induced brain injury is unclear; however, research indicates that astrocytes become hypertrophic and overexpress GFAP following irradiation [43, 44]. GFAP protein levels increase in the rat and mouse brain both acutely, 24 h post-irradiation, and chronically, 4-5 months post-irradiation [19, 20]. Astrocytes also have been shown to overexpress MCP-1 following irradiation of a rat brain [43]. Furthermore, cultures of astrocytes irradiated with a single dose of 25 or 35 Gy display increased Cox-2 message levels; however, at lower more clinically relevant doses, a significant increase in Cox-2 has not been observed [44]. Interestingly, conditioned media from irradiated microglial cells induced astrogliosis in vitro, which may exacerbate radiation-induced inflammation [66].

Many CNS disorders are associated with astrogliosis. During astrogliosis, astrocytes are thought to increase the production of proinflammatory mediators, such as arachidonic acid, Cox-
2, MCP-1, and IL-1β [49, 67-69]. Arachidonic acid, which is a substrate for Cox-1 and Cox-2, is released by phospholipase A₂ (PLA₂). PLA₂, Cox-2, MCP-1, and IL-1β have been associated with neurodegenerative diseases [68-71]. Interestingly, both secretory PLA₂ (sPLA₂) and cytosolic PLA₂ (cPLA₂) contain a PPRE in their promoters. In primary rat brain astrocytes, pretreatment of astrocytes with L-165041 attenuated LPS-induced sPLA₂ expression [72]. Studies in our lab indicate that incubating primary rat astrocytes with the PPARδ agonist, L-165041, inhibits the radiation-induced increase in GFAP message and protein levels, as well as the increase in IL-6 and MCP-1 message levels. This occurs, in part, by preventing the activation of the PKCα/MEK1/2/ERK1/2 pathway. These data support the hypothesis that PPARδ activation can modulate the radiation-induced proinflammatory response in astrocytes.

Oligodendrocytes are terminally differentiated glial cells that produce myelin sheath for the axons of neurons. Oligodendrocytes arise from oligodendrocyte-2 A (O-2A) progenitor cells. Irradiation of the rat brain and spinal cord reduces the reproductive capacity of the O-2A progenitor cells, which in turn, decreases the production of new oligodendrocytes [73]. This decreased production of oligodendrocytes might contribute to radiation-induced demyelination [73, 74]. Mature oligodendrocytes are also radiosensitive and undergo radiation-induced apoptosis [75]. Clinical brain white matter specimens obtained post-radiation demonstrate early loss of oligodendrocyte progenitors [76]. Ultrastructural analysis of rat brains indicates that progressive degradation of myelin sheaths with axonal preservation occurs 15 months following 25 Gy WBI [76]. Radiation-induced oligodendrocyte cell death is thought to occur via p53-dependent apoptosis [77]. TNF-α released from irradiated microglia and astrocytes also may contribute to radiation-induced oligodendrocyte cell death [77, 78]. Our data, which demonstrate that PPARδ modulates radiation-induced inflammation in microglia and astrocytes, suggest that PPARδ may, in turn, ameliorate radiation-induced oligodendrocyte cell death. Although studies have not investigated if PPARδ activation ameliorates radiation-induced oligodendrocyte cell death, PPARδ agonists have ameliorated disease progression in a mouse
model of multiple sclerosis, a demyelinating disease, by reducing inflammatory cell activation [17].

Although oligodendrocyte apoptosis has been observed following large single doses of irradiation, qualitative observation in rats 12 months following 45 Gy fWBI did not reveal white matter necrosis, or changes in the size of major forebrain commissures, the number of oligodendrocytes, the size and number of myelinated axons, or the thickness of myelin [79]. Although no gross morphology or structural alterations of myelin were observed, advanced imaging techniques such as diffusion tensor imaging (DTI) may be required to observe changes in white matter following irradiation. DTI is a technique used for examining changes in the microstructure of white matter [80]. These changes have been correlated to cognitive outcomes. In particular, decreased fractional anisotropy (FA) and increased apparent diffusion coefficient (ADC) are related to lower intellectual outcome in children treated with cranial-spinal radiation for medulloblastoma relative to age-matched controls [81]. In accord with these findings, the FA is decreased in small cell lung cancer patients who received prophylactic cranial irradiation [82]. These findings, which indicate there is a correlation between compromised fiber integrity, tissue loss, and lower IQ, are consistent with previous findings that white matter volume loss is related to adverse intelligence and academic outcome [83].

Whether PPARδ activation can inhibit alterations in white matter following irradiation is unknown; however, experimental data demonstrate that PPARδ may play a role in myelogenesis. A possible role for PPARδ and myelogenesis was first illustrated in PPARδ-KO mice; these mice display a defect in the myelination of their corpus callosum [84]. Further implicating PPARδ in myelogenesis, bromopalmitate, a weak PPARδ agonist, increased the percentage and size of sheet-bearing oligodendrocytes produced when applied to primary mouse glial cultures [85]. In accord with these findings, a more selective PPARδ agonist, L-796449, has been shown to accelerate differentiation of oligodendrocytes within 24 h of being added to primary mouse glial cultures. This included the number of oligodendrocytes with
processes and membrane sheets, the size of the membrane sheets, and the distribution and intensity of mRNA levels of MBP and PLP [86]. In addition, in OPCs generated from E13 mice, GW0742 increased the number of MBP stained cells and increased mRNA levels of OPC markers of maturation, including Oligo1, CGT, PDGFRα, and GALC [87]. GW0742 also increased staining of the bone morphogenetic protein (BMP) antagonist, noggin, and decreased mRNA levels of BMP2 and BMP4, which are known to inhibit OPC differentiation [87]. Furthermore, preliminary studies in our lab demonstrate that PPARδ KO mice display decreased MBP expression following irradiation.

Similar to oligodendrocytes, studies have failed to detect overt changes in the number of mature neurons in rats following 45 Gy fWBI; however, radiation does alter neuronal gene expression and neuronal function [88]. Changes in gene expression induced by learning that alter the composition of hippocampal neuronal networks and contribute to synaptic plasticity and synaptic strength have been observed following irradiation. In particular, rats irradiated with 45 Gy fWBI exhibited changes in N-methyl-D-aspartic acid (NMDA) receptor subunits, which are important for synaptic transmission and plasticity [89]. Additionally, mice irradiated with 10 Gy WBI displayed altered expression of the activity-regulated cytoskeleton-associated protein (Arc), which is expressed following learning tasks and plays a role in modulating hippocampal synaptic plasticity [90-92]. Furthermore, whole-body radiation alters the LTP in the hippocampus in several rodent models [93, 95]. Since inflammation alters NMDA expression, Arc expression and LTP, it is possible that the anti-inflammatory properties of PPARδ can restore the impaired molecular and functional properties of neurons following irradiation [97-103].

Endothelial cell dysfunction may contribute to radiation-induced brain injury by: i) facilitating the breakdown of BBB, and ii) increasing the proinflammatory response. Microvascular endothelial networks, which are important for the supply of oxygen and nutrients, are the most radiosensitive part of the vasculature [104]. Endothelial cell dysfunction following irradiation is characterized by arterial wall thickening and hyalinization [105, 106]. The vascular
hypothesis is a traditional view that ischemia-induced endothelial cell loss is the primary driving force of radiation-induced brain injury. The radiation-induced disruption of the neuron-vasculature interaction also may contribute to the damaging effect of WBI on the neurogenic niche and hippocampal neurogenesis [11]. PPARδ has been shown to promote endothelial cell survival after ischemic insults. In particular, GW501516 significantly reduced cerebral vascular endothelial cell degeneration in an OGD mouse model. GW501516 protected against vascular cell death by inhibiting expression of miR-15a, which directly regulates the anti-apoptotic protein Bcl-2. This resulted in increased Bcl-2 protein expression, reduced Golgi fragmentation, and decreased caspase-3 activity, which in turn reduced cerebrovascular permeability and infarct volume in vivo [107]. Thus, it is possible that PPARδ can promote endothelial cell survival following WBI.

In addition to microglia and astrocytes, brain endothelial cells produce a proinflammatory response following irradiation. In the rat brain microvasculature, WBI increased the expression of the adhesion molecules, ICAM-1, E-selectin, and P-selectin [108]. Studies also indicate that irradiation induces ICAM-1 in human umbilical vein endothelial cells (HUVEC) [109]. Furthermore, irradiation of immortalized rat brain microvascular endothelial cells, RBMEC-GP8.3 cells, leads to increased: i) intracellular ROS generation, ii) activation of NF-κB, and iii) expression of ICAM-1 and PAI-1 [110]. Given the anti-inflammatory actions of PPARδ activation in microglia and astrocytes, it is likely that PPARδ can also modulate the radiation-induced increase in proinflammatory mediators in endothelial cells.

In summary, the discussion above indicates that radiation-induced brain injury is not simply due to a single cell population. Instead, radiation-induced brain injury is a multicellular process. Based on the findings described above, we propose a model for the role of PPARδ in the modulation of radiation-induced brain injury. Irradiating the brain leads to increased microglial activation, characterized by the activation of the stress-activated kinases, PKCo, MEK1/2, and ERK1/2, and the proinflammatory transcription factors, NF-κB and AP-1.
Activation of these transcription factors increases the expression of proinflammatory mediators, including Cox-2, MCP-1, IL-1β, and TNF-α. These proinflammatory mediators alter the brain microenvironment and, in turn, alter the function of astrocytes, oligodendrocytes, endothelial cells, and neurons. Activation of PPARδ prevents the radiation-induced increase in microglial activation, and thus, may restore partially the microenvironment of the brain. Therefore, PPARδ activation represents a novel therapeutic strategy to mitigate the radiation-induced increase in inflammatory markers of brain injury.
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CHAPTER VI
FUTURE DIRECTIONS

Our *in vitro* studies demonstrated that the PPARδ agonist, L-165041, inhibited the radiation-induced increase in: i) intracellular ROS generation, ii) Cox-2 and MCP-1 expression, and iii) IL-1β and TNF-α message levels in BV-2 murine microglial cells. PPARδ activation prevented the radiation-induced proinflammatory response, in part, through the inhibition of intracellular ROS generation, which in turn, prevented activation of the PKCa/MEK1/2/ERK1/2/AP-1 pathway. Although using BV-2 cells offers many advantages, such as the relative ease of experimental procedures and design, there are also several disadvantages to using these cells. In particular, BV-2 cells are a transformed cell line generated by infecting primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus [1]. BV-2 cells, thus, are unlikely to represent fully primary microglia in the brain. Therefore, in addition to our cell signaling work in BV-2 cells, we examined whether PPARδ activation prevented the radiation-induced increase in microglial activation *in vivo*. We observed that administering the PPARδ agonist, GW0742, prevented the increase in microglial activation (CD68+ cells) in the DG 1 week following irradiation. We attempted to examine the expression of proinflammatory mediators, including Cox-2, in CD68+ cells following irradiation; however, this method proved challenging. For example, although we could quantify the number of cells expressing Cox-2, the relative amount of Cox-2 expression in each cell could not be quantified.

Future studies should, therefore, extend our *in vitro* findings by using flow cytometric analysis that enables the examination of the radiation-induced proinflammatory response in microglia *in vivo*, and to determine whether PPARδ activation can modulate this response [2]. In this model, rodent brains would be irradiated and proinflammatory markers in isolated microglia would be assessed using flow cytometric analysis. In the adult rodent, peripheral macrophages and microglia express the protein tyrosine phosphatase, CD45. The function of
CD45 is not understood fully; however, it does act as an inhibitory receptor on cells of the myeloid origin [1]. Microglia are the only macrophage population that express a log order lower level of CD45 [1, 3-6]. Thus, resident microglia can be sorted from infiltrating macrophages by their relative CD45 expression [1, 3-6]. When microglia become activated they express higher levels of CD45; however, they generally remain lower than the CD45 levels on populations of unactivated peripheral macrophage [3-6]. Flow cytometric analysis is, therefore, a powerful tool because it will help elucidate the radiation response of both resident microglia and infiltrating macrophages, as well as the response of highly activated microglia and unactivated microglia.

The radiation response of primary microglia has not been examined adequately by flow cytometric analysis. Thus, a time course of the radiation-induced proinflammatory response in resident microglia and infiltrating macrophages needs to be determined. Additionally, whether radiation increases the phosphorylation of PKCα, MEK1/2, and ERK1/2 in microglia in vivo could be addressed to determine if similar pathways are activated in BV-2 cells and primary microglia following irradiation. After establishing the radiation-induced response in primary microglia, it would be of interest to examine whether PPARδ can modulate these responses. Additionally, since we failed to detect an increase in CD68+ cells in PPARδ knockout (KO) mice, future studies should determine if microglia isolated from PPARδ KO mice exhibit a different proinflammatory response following irradiation compared to wildtype (WT) mice.

Whether PPARδ agonists alternatively activate primary microglia from the proinflammatory M1 phenotype to the anti-inflammatory M2 phenotype following irradiation also could be addressed by future studies. The M1 phenotype is the “classical activation” of microglia, which is associated with the proinflammatory phase of the innate immune response. This state of microglial activation is associated with the production of proinflammatory cytokines, such as TNF-α, IL-6, IL-1β, proteases, nitric oxide, and ROS [7-10]. Following the proinflammatory response of the innate immune system is the anti-inflammatory repair phase that leads to wound healing and tissue homeostasis [7, 11-13]. There are four main anti-
inflammatory cytokines involved in tissue repair and wound healing, including IL-4, IL-13, IL-10, and TGF-β [7, 11-13]. In this phase, microglia are categorized as “alternatively activated” or “deactivated”. The alternative activation of microglia generally is induced by the cytokines IL-4 or IL-13 and is associated with genes that promote tissue repair and reconstruction of the extracellular matrix. In contrast, the acquired deactivation of microglia is induced by the cytokines IL-10 or TGF-β and is associated with the uptake of apoptotic cells. The term M2 is often used to describe both the alternative activation and the acquired deactivation of microglia [7]. Markers of the M2 phenotype include: arginase 1 (Arg1), found in inflammatory zone 1 (Fizz1), mannose receptor 1 (Mrc1), c-type lectin domain family 7 member A/Dectin (Clec7a), jagged1 (Jag1), resistin like alpha (Retnla), programmed cell death 1 ligand 2 (Pdcd1lg2), and chitinase 3-like 3 (Chi3l3/Ym1) [14].

PPARδ has been shown to alternatively activate macrophages from the proinflammatory M1 phenotype to the anti-inflammatory M2 phenotype. In particular, PPARδ controls the alternative activation of adipose tissue macrophages (ATMs) and Kupffer cells, hepatic macrophages [15, 16]. In the white adipose tissue of PPARδ KO mice, genes of alternatively activated macrophages, Clec7a, Retnla, Tgfb1, Jag1, and Mrc1, were reduced. The alternative activation of Kupffer cells also was decreased in PPARδ KO mice, as assessed by reduced expression of Arg1, Clec7a, Jag1, Pdcd1lg2, and Chia [15]. Reduced alternative activation of ATMs and Kupffer cells in PPARδ KO mice led to impaired glucose tolerance and insulin resistance [15, 16]. Interestingly, PPARδ expression is thought to alternatively activate microglia from the M1 phenotype to the M2 phenotype in steroid receptor coactivator-3 (SRC-3) KO mice with experimental autoimmune encephalomyelitis in their spinal cord. In SRC-3 KO mice there was a correlation between PPARδ expression and ramified microglia that expressed Chi3l3/Ym1/2 and Mrc1, phenotypic markers for alternative activation [17]. Whether PPARδ agonists can alternatively activate microglia remains to be determined. Future work should extend our findings that GW0742 prevents the radiation-induced increase in cells expressing
CD68, a lysosomal protein often used as a marker of microglial activation, to examine whether PPARδ agonists promote an increase in M2 microglia. In this model, rodent brains would be irradiated, microglia would be isolated using fluorescence assisted cell sorting, and RNA would be harvested to examine the expression of markers of the M2 phenotype.

Similar to microglia, astrocytes once were regarded solely as passive bystanders in the brain. Research now indicates that integrated signaling circuits between astrocytes and neurons exist, and further, that the dysregulation between these signaling networks is involved in the pathogenesis of neurological disorders, including amyotrophic lateral sclerosis (ALS), epilepsy, and stroke [18-21]. A key function of astrocytes is to remove neurotransmitters, such as glutamate, released by neurons to prevent excitotoxicity induced by excessive stimulation of neurons. Astrocytes uptake glutamate via two transporters, including the glutamate-aspartate transporter (GLAST), known as the Excitatory Amino Acid Transporter 1 (EAAT1) in humans, and the glutamate transporter 1 (GLT1), known as EAAT2 in humans [22, 23]. Astrocytes also can influence neurons by releasing neuroactive agents, such as glutamate, ATP, and serine. Although the exact mechanisms underlying the release of gliotransmitters are unclear, studies suggest that astrocytes may receive synaptic input from glutamatergic and gamma-aminobutyric acid (GABA)-containing neurons [18]. It has been proposed that the function of gliotransmitter release is to modulate the strength of inhibitory and excitatory synaptic transmission by activating receptors on neurons [24]. Of note, astrocyte processes can reach thousands of synapses simultaneously; thus, gliotransmitter release may promote rapid synchronization of synaptic transmission [18]. Evidence suggests that disturbed glutamate uptake and release by astrocytes is involved in the pathogenesis of some neurological disorders. For example, increased glutamate has been observed in epileptogenic foci [25]. Additionally, reactive astrocytes in the spinal cord of patients with ALS display increased immunoreactivity for metabotropic glutamate receptors, including mGluR1α, mGluR5, and mGluR2/3, which are key receptors involved in the release of glutamate from astrocytes [26]. Furthermore, stroke-
induced neuronal damage caused by excitotoxicity in different brain regions has been attributed to the differential expression of GLAST and GLT1 [27].

The role of astrocytes in the progression of radiation-induced brain injury is unclear; future studies could examine whether astrocyte functionality is altered following irradiation. Since glutamate is an important neurotransmitter involved in synaptic plasticity, learning, and memory, it is possible that dysregulation of glutamate contributes to radiation-induced cognitive impairment. Future studies, therefore, could examine extracellular glutamate release by astrocytes following irradiation of primary astrocytes in vitro. Radiation also may alter the expression of receptors involved in glutamate regulation, thereby contributing to radiation-induced brain injury by disturbing the communication between astrocytes and neurons. Thus, future studies could examine the expression of glutamate receptors both in vitro in primary astrocytes and in vivo in the rodent brain.

If changes in glutamate or glutamate transporters are detected following irradiation, studies should examine whether PPARδ activation can modulate these changes. Although it is unknown if PPARδ modulates glutamate uptake or release, numerous studies have demonstrated that the neuroprotective efficacy of PPARγ agonists is mediated, in part, by regulating the glutamatergic pathway. For instance, the PPARγ agonist, rosiglitazone, prevented stress-induced defects in glutamate uptake in young-adult rats by restoring expression of GLT1 [28]. In addition, the PPARγ agonist, pioglitazone, significantly improved memory impairment induced by scopolamine by modulating the glutamatergic pathway [29]. These studies provide a rationale for investigating whether PPARδ activation regulates the glutamatergic pathway, similar to PPARγ. Since our in vivo findings highlighted the possible importance of activated astrocytes, rather than microglia, in the radiation response of PPARδ KO mice, it would also be interesting to compare the radiation-induced changes of glutamate signaling in astrocytes isolated from WT and KO mice.
Given that PPARδ activation modulates markers of inflammation both in vitro and in vivo, we hypothesized that PPARδ activation would mitigate radiation-induced cognitive impairment. WBI leads to a progressive deterioration of both hippocampal- and non-hippocampal dependent cognitive function [30]. The Barnes Maze task is a measure of hippocampal-dependent cognitive function, including spatial memory retention. Several studies have demonstrated that the Barnes Maze task is a sensitive task to detect radiation-induced cognitive impairment in C57Bl/6 mice. For example, Raber et al. demonstrated that 2-month old C57Bl/6 mice administered 10 Gy of X-irradiation showed hippocampal dependent deficits as early as 3 months post-irradiation when performing the Barnes Maze task [31]. In addition, Wong-Goodrich et al. revealed that 8-week old female C57Bl/6 mice irradiated with a single dose of 5 Gy displayed cognitive impairment on the Barnes Maze task [32]. We detected cognitive impairment 3 months following WBI using the Barnes Maze task; however, these deficits were no longer present by 6 months post-WBI, suggesting that the impairments were an early delayed response rather than progressive cognitive impairment. The Barnes Maze task is a new cognitive task in our lab; therefore, we could try to modify the task in an attempt to make it more sensitive in detecting radiation-induced cognitive impairment. In particular, Wong-Goodrich’s group performed all cognitive testing during the dark phase of the 12/12-h light/dark cycle. Since mice are nocturnal species, and most of their activity occurs during the dark phase of the circadian cycle, testing mice on the dark phase of the light/dark cycle may have an effect on their performance on the visuo-spatial-dependent Barnes Maze task [33]. Given that we observed many of the mice hesitated to enter the escape box, we could try using the modified Barnes Maze, which has the escape holes located in a wall surrounding the circular arena rather than on the floor of the circular arena. Thus, mice would not have to climb down into a hole, which they appeared hesitant to do [34].

Interestingly, our lab also has been unsuccessful in detecting hippocampal-dependent cognitive dysfunction in young adult male rats administered 40 Gy fractionated WBI (fWBI) 6
months to 1 year following irradiation. Our lab has, however, developed an experimental rat model of radiation-induced brain injury to measure hippocampal-independent cognitive function using the novel object recognition (NOR) task. In this model, we observe cognitive impairment 6 months to 1 year after fractionated WBI [35]. Our lab has demonstrated successfully that administration of i) pioglitazone, a PPARγ agonist; ii) fenofibrate, a PPARα agonist; iii) L-158,803, an angiotensin type I receptor antagonist (AT1RA); or iv) ramipril, an angiotensin-converting enzyme inhibitor, prevents and/or ameliorates WBI-induced cognitive impairment using the NOR task [35, 36]. Although GW0742 does not protect against early delayed changes in cognition, it is possible that the mechanisms underlying early delayed cognitive impairment are different than those contributing to progressive cognitive impairment. Future studies, therefore, could examine if administration of GW0742 ameliorates and/or prevents radiation-induced cognitive impairment in young adult male rats using the NOR task.

Future studies could also determine if GW0742 administration prevents radiation-induced cognitive impairment in very young (pediatric), middle-aged, or older animals. Decreased hippocampal neurogenesis has been hypothesized to play a key role in WBI-induced cognitive impairment in young animals; however, hippocampal neurogenesis may play a minor role in the radiation response of middle- and old-aged brains [37, 38]. Hippocampal neurogenesis is reduced progressively with age in rats and mice [39, 40]. Interestingly, WBI decreased the number of immature neurons in young rats (8 weeks old), but not in middle-aged (18 months old) or old rats (28 months old). Furthermore, microglial activation is increased markedly in older animals compared to young adult animals [18]. Thus, the brain microenvironment is different in older animals compared to younger ones, and the age at the time of WBI may be a critical factor in determining the mechanisms underlying radiation-induced brain injury. It is, therefore, likely that in older animals a decrease in hippocampal neurogenesis does not contribute significantly to radiation-induced cognitive impairment. Although GW0742 did not prevent the radiation-induced decrease in hippocampal neurogenesis, the PPARδ
agonist did prevent the increase in activated microglia following irradiation. Therefore, GW0742 may be an efficacious agent to prevent radiation-induced brain injury in older individuals. Future studies could, therefore, examine whether GW0742 administration modulates the WBI-induced cognitive impairment in rodents of different age groups. These studies would be important since: i) pediatric cancer patients have a higher long-term survival rate (up to 80%); ii) the risk of developing brain metastases increases during or after middle age; and iii) patients older than 60 years of age are at a higher risk for developing radiation-induced dementia [41-45].

If future studies determine that administration of GW0742 mitigates progressive cognitive impairment or cognitive deficits in rodents of different age groups, studies could focus on better understanding the neuroprotective mechanism of PPARδ. Neuroinflammation, in addition to altering hippocampal neurogenesis, has been proposed to alter the function of pre-existing neurons. Since administration of GW0742 inhibited markers of inflammation following WBI, we could examine the effect of the PPARδ agonist on the synaptic level. Long-term potentiation (LTP) induced by high-frequency stimulation in the hippocampus is thought to underlie learning and memory; it is a measure of signal transmission between two neurons [46, 47]. Radiation induces defects in the LTP of synaptic transmission in the hippocampus of rodents [48]. This is thought to occur, in part, via the radiation-induced increase in ROS and inflammatory cytokines, such as IL-1B. For example, guinea pigs exposed to γ-irradiation or \( \text{H}_2\text{O}_2 \) had impaired LTP in CA1 slices [49-51]. Additionally, LTP was altered in the dentate gyrus of rats that were administered \( \text{H}_2\text{O}_2 \) intracerebroventricularly and in IL-1β-treated rats [52, 53]. Lonergan et al. demonstrated that eicosapentaenoic acid, a poly-unsaturated fatty acid with anti-inflammatory properties, restored the excitatory post-synaptic potential (EPSP) in irradiated animals [48]. Future studies, therefore, could determine if administration of GW0742 can protect against radiation-induced alterations in LTP. These studies would help elucidate the neuroprotective efficacy of PPARδ activation.
Historically, radiation-induced brain injury has been studied in the absence of a gross tumor [31, 12-15]. Since the presence of a brain tumor may worsen cognitive impairment and contribute to increased oxidative stress and inflammatory mediators in the brain, an orthotopic brain tumor model should be established to examine whether administration of GW0742 can modulate WBI-induced brain injury in a tumor bearing model [54-56]. These studies would also be important because the role of PPARδ and cancer progression is controversial. There are a number of contradictory studies in the literature regarding the role of PPARδ in cancer; however, two main hypotheses exist [57-60]. These include: i) PPARδ is overexpressed in tumors, promotes anti-apoptotic signaling and cell proliferation, and thus, enhances tumorigenesis; and ii) PPARδ promotes anti-inflammatory signaling and terminal differentiation, thereby inhibiting tumor growth [61].

Although the role of PPARδ in cancer progression is controversial, evidence suggests that PPARδ promotes differentiation of neuroblastomas, pediatric tumors that originate from immature neuroblasts. Differentiation therapy is a therapeutic approach aimed at forcing tumor cells to undergo maturation and differentiation. Undifferentiated cells often lack growth restraints, and therefore, the goal of this approach is to control the growth rate of tumor cells in order to slow down their progression. Oleic acid has been shown to promote differentiation of a variety of neuroblastoma cell lines, including SH-SY5Y cells. Many of the effects of oleic acid are thought to be mediated by PPARδ. Treatment of SH-SY5Y cells with GW0742 promoted cell cycle arrest and decreased migration and invasiveness. GW0742 also increased neuronal differentiation as demonstrated by increased i) neurite outgrowth, and ii) expression of neuronal markers including substance P, GAP-43, N-CAM, neurofilament-200, and neuronal specific enolase [62, 64].

In addition to neuroblastoma differentiation, PPARδ may aid in the differentiation of rapidly growing glioma cells to oligodendrocytes. In particular, PPARδ overexpression was demonstrated to upregulate PPARγ in rat C6 glioma cells. This subsequently resulted in
increased oligodendrocyte-specific markers, including the myelin basic protein and alkyl-dihydroxyacetone phosphate synthase (ADAPC), an enzyme involved in synthesis of myelin-rich plasmalogens. It also resulted in decreased GFAP expression, a stem cell marker [65]. Studies investigating the effects of PPARδ on brain tumor growth are limited and need to be expanded to include primary and metastatic brain tumor bearing mouse models. 

In this thesis, we focused on modulating radiation-induced brain injury by mitigating inflammatory responses following irradiation; however, mechanisms other than neuroinflammation are likely to contribute to radiation-induced cognitive impairment. Therefore, future studies could focus on understanding the mechanisms underlying radiation-induced brain injury. DNA methylation is an important epigenetic mechanism for controlling gene expression and gene silencing. Altered DNA methylation contributes to the progression of a variety of neurological diseases, including Alzheimer’s and Parkinson’s disease [80, 82]. Whether radiation-induced late delayed brain injury involves epigenetic changes has not been examined; however, recent studies have begun to investigate whether radiation can alter DNA methylation. Koturbash et al. demonstrated that DNA methylation was decreased in irradiated cutaneous tissue 6 hours following exposure to 1 Gy of x-rays [68]. In addition, MCF7 breast cancer cells irradiated with 10 Gy delivered in 2 Gy fractions exhibited locus-specific alterations in DNA methylation [78]. Furthermore, 5 Gy acute or fractionated x-rays led to decreased global DNA methylation in the thymus of C57Bl mice 1 month after irradiation, suggesting that radiation may lead to persistent changes in DNA methylation [79].

To examine radiation-induced alterations in DNA methylation in the brain, we could use the HpaII-based cytosine extension assay, which measures the proportion of unmethylated CCGG sites in genomic DNA [68]. To determine genes differentially methylated in non-irradiated versus irradiated brains, we could immunoprecipitate methyl-CpG sites and then profile methylated genes using CpG island microarrays [78]. If DNA methylation is altered following irradiation in the brain, the mechanism of methylation change should be investigated.
Three DNA methyltransferases, DNMT1, DNMT3a and DNMT3b, are involved in establishing and maintaining DNA methylation patterns at CpG sites [69-71]. The methyl CpG-binding domain (MBD) family of proteins mediates the association of DNA methylation with transcriptional repressions. MBD proteins that interact with methylated DNA and play a role in methylation-mediated chromatin remodeling and gene silencing, include MeCP2, MBD1, MBD2, MBD3 [72-77]. To investigate the mechanism of methylation change, the expression of i) DNMT1, DNMT3a, and DNMT3b methyltransferases, and ii) MBD proteins could be examined.
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PUBLICATIONS


Schnegg CI*, Kooshki M*, Hsu FC, Robbins ME. PPARδ modulates the radiation-induced proinflammatory response in primary rat astrocytes by preventing activation of
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Kooshki M*, Schnegg CI*, Green-Schloesser D, Payne VS, Hsu FC, Robbins ME. The PPARδ agonist, GW0742, inhibits microglial activation and inflammation but does not restore neurogenesis after mouse whole-brain irradiation. (Submission to Neuro-Oncology: June 2012)(* denotes equal contribution)

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