Published in final edited form as:

Metab Brain Dis. 2016 June ; 31(3): 683–692. doi:10.1007/s11011-016-9805-2.

Loss of PPAR α perpetuates sex differences in stroke reflected by peripheral immune mechanisms

Abby L. Dotson^{1,2}, Jianming Wang³, Jian Liang³, Ha Nguyen^{1,2}, Dustin Manning^{1,2}, Julie A. Saugstad³, and Halina Offner^{1,2,3,*}

¹Neuroimmunology Research, VA Medical Center, Portland, OR, USA

²Department of Neurology, Oregon Health & Science University, Portland, OR, USA

³Department of Anesthesiology & Perioperative Medicine, Oregon Health & Science University, Portland, OR, USA

Abstract

Peroxisome proliferator-activated receptor alpha (PPAR α) is a nuclear receptor transcription factor that plays a role in immune regulation. Because of its expression in cerebral tissue and immune cells, PPAR α has been examined as an important regulator in immune-based neurological diseases. Many studies have indicated that pre-treatment of animals with PPAR α agonists induces protection against stroke. However, our previous reports indicate that protection is only in males, not females, and can be attributed to different PPAR α expression between the sexes. In the current study, we examine how loss of PPAR α affects male and female mice in experimental stroke. Male and female PPAR α knockout mice were subject to middle cerebral artery occlusion (MCAO) or sham surgery, and the ischemic (local) or spleen specific (peripheral) immune response was examined 96 hours after reperfusion. We found that loss of PPAR α perpetuated sex differences in stroke, and this was driven by the peripheral, not local, immune response. Specifically we observed an increase in peripheral pro-inflammatory and adhesion molecule gene expression in PPAR α KO males after MCAO compared to females. Our data supports previous evidence that PPAR α plays an important role in sex differences in the immune response to disease, including stroke.

Keywords

Experimental stroke; Sex difference; Inflammation; PPAR α ; Knock Out

*Corresponding author: Dr. Halina Offner, Neuroimmunology Research, R&D-31, Portland Veterans Affairs Medical Center, 3710 SW US Veterans Hospital Rd., Portland, OR 97239, USA. Fax: +1-503-721-7975; offnerva@ohsu.edu.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of Oregon Health & Science University's Institutional Animal Care and Use Committee.

This article does not contain any studies with human participants performed by any of the authors.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptors that function as transcription factors. PPARs are activated by their natural ligands, lipids and metabolites. Activation leads to binding of PPAR to Retinoid X Receptor (RXR), another nuclear receptor, forming a heterodimer that in turn binds to the PPAR Response Element (PPRE), a DNA response element localized in promoter regions of energy and lipid metabolism genes (Bishop-Bailey 2000). Immunohistochemical studies illustrate distinct patterns of PPAR expression in the brain depending on the area and the cell population (Moreno et al. 2004). Specifically, PPAR α is expressed in defined brain areas, predominantly in cortical, striatal and thalamic regions.

The role of PPAR α in the immune response has been relatively well studied. Similar to the other isoforms, PPAR α is a nuclear hormone receptor that regulates lipid metabolism in the liver. Additionally, PPAR α negatively regulates inflammation. PPAR α ligands interfere with the NF- κ B signaling pathway, thereby inhibiting the production of pro-inflammatory cytokines and reducing oxidative stress (Delerive et al. 2001). PPAR α also plays a role in leukocyte-endothelial adhesion (Marx et al. 1999; Poynter and Daynes 1998). PPAR α is expressed in neurons and astrocytes in the brain as well as cerebral vascular endothelial (Bishop-Bailey 2000; Cullingford et al. 1998; Kainu et al. 1994). Peripheral PPAR α expression is detected in the liver and in secondary (peripheral) immune organs such as the Peyer's patch and the white pulp of the spleen (Braissant et al. 1996; Moreno et al. 2004). PPAR α is also expressed in certain immune cell subsets, specifically, monocytes/macrophages and lymphocytes (Chinetti et al. 1998; Jones et al. 2002). Because of its cerebral and cellular expression and its role in inflammation, oxidative stress and leukocyte extravasation, PPAR α has been examined as an important regulator in immune-based neurological diseases.

Stroke is still a primary cause of death and disability in the United States (Mozaffarian et al. 2015). The immune response is a key factor in stroke outcome. Peripheral leukocytes can infiltrate the brain after ischemic injury, thereby contributing to neurodegeneration and infarct volume (Offner et al. 2006a; Offner et al. 2006b; Seifert et al. 2012a; Seifert et al. 2012b). Males respond differently to stroke than females, and the spleen plays an important role in those outcomes. In general, male mice exhibit an early increase in ischemic damage following experimental stroke that is resolved without the presence of the spleen (Dotson et al. 2015b). Activated monocytes/microglia in the brain and peripheral CD11b+ cells are key components of stroke sex differences as they are more prevalent in male mice after MCAO and their loss eliminates sex differences, respectively (Banerjee et al. 2013; Dotson et al. 2015b).

Although females initially exhibit a more favorable stroke outcome, they often suffer from more long-term stroke effects, including disability that lead to poorer quality of life. (Di Carlo et al. 2003). In addition to basic sex differences in response to stroke, the discrepancy in translation of bench research to clinical success in stroke therapy can be attributed, in part, to underrepresentation of females in therapy-based basic research (Turner et al. 2013).

For these reasons, it is imperative that both sexes are studied when identifying the molecular contributors to stroke.

Our previous study identified sex differences in stroke outcome with activation of PPAR α (Dotson et al. 2015a). We observed that a PPAR α agonist did not protect females during ischemic stroke as it did in males and those disparities were likely the result of slight alteration of gene expression in the brain and peripheral immune cell subsets (Dotson et al. 2015a). In the present study, we compared outcomes in male and female PPAR α knockout (KO) mice 96 hours after MCAO. We also compared male and female PPAR α KO MCAO mice to WT MCAO and sham PPAR α KO mice. We found that loss of PPAR α perpetuated sex difference in stroke outcome. The peripheral immune response, characterized by an increase in pro-inflammatory and adhesion molecule gene expression in MCAO male PPAR α mice, was predominately responsible for the observed sex differences after MCAO.

Materials and Methods

Ethics Statement

The study was conducted in accordance with National Institutes of Health guidelines for the use of laboratory animals, and the protocols were approved by the Portland Veteran Affairs Medical Center Institutional Animal Care and Use Committee, local database ID # 2840 and the Oregon Health and Science University Animal Care and Use Committee, protocol # IS00003885.

Experimental Animals

Male and female C57BL/6J wild-type (WT) mice or PPAR α knockout (KO) mice (The Jackson Laboratory, Sacramento, CA, USA) were housed in a climate-controlled room on a 12-hour light/dark cycle. Mice were used at a weight of 20–25g, which correlated with an age of 8–10 weeks for males and 10–12 weeks for females. Food and water were provided ad libitum.

Middle Cerebral Artery Occlusion Model

Focal cerebral ischemia was induced in male and female mice for 1 hour by transient, reversible right middle cerebral artery occlusion (MCAO) under isoflurane anesthesia followed by 96 hours of reperfusion as described previously (Zhang et al. 2008). Head and body temperature were controlled at $37.0 \pm 1.0^{\circ}\text{C}$ throughout MCAO surgery with a warm water blanket and a heating lamp ~18 inches above the mouse. Occlusion and reperfusion were verified in each animal by Laser Doppler Flowmetry (LDF) (Model DRT4, Moor Instruments Ltd., Wilmington, DE). The common carotid artery was exposed and the external carotid artery was ligated and cauterized. Unilateral MCAO was accomplished by inserting a 6-0 nylon monofilament surgical suture (ETHICON, Inc., Somerville, NJ, USA) with a heat-rounded and silicone-coated (Xantopren comfort light, Heraeus, Germany) tip into the internal carotid artery via the external carotid artery stump. Adequacy of MCAO was confirmed by monitoring cortical blood flow at the onset of the occlusion with a LDF probe affixed to the skull. Animals were excluded if mean intra-ischemic LDF was greater than 30% pre-ischemic baseline LDF. At 1 hour of occlusion, the occluding filament was

withdrawn to allow for reperfusion. Mice were then allowed to recover from anesthesia and survived for 96 hours of reperfusion.

Infarct Volume Analysis

Brains were harvested after 96 hours of reperfusion and sliced into five 2-mm-thick coronal sections for staining with 1.2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) in saline as described previously (Hurn et al. 2007). The 2-mm brain sections were incubated in 1.2% TTC for 15 min at 37°C, and then fixed in 10% formalin for 24 hours. Infarct volume was measured using digital imaging and image analysis software (Systat, Inc., Point Richmond, CA, USA). To control for edema, infarct volume (cortex, striatum, and hemisphere) was determined by subtraction of the ipsilateral non-infarcted regional volume from the contralateral regional volume. This value was then divided by the contralateral regional volume and multiplied by 100 to yield regional infarct volume as a percent of the contralateral region.

Neurological deficit scores

Neurological deficit scores were determined at baseline, after 5 minutes of reperfusion, then at 1, 2, 3, and 4 days post-occlusion (POD) to confirm ischemia and the presence of ischemic injury. We used a 0 to 5 point scale as follows: 0, no neurological dysfunction; 1, failure to extend left forelimb fully when lifted by tail; 2, circling to the contralateral side; 3, falling to the left; 4, no spontaneous movement or in a comatose state; and 5, death. Any animal without a deficit at POD1 was excluded from the study.

Leukocyte isolation from brains and spleen

Spleens from individual mice were removed and a single-cell suspension was prepared by passing the tissue through a 100 µm nylon mesh (BD Falcon, Bedford, MA). The cells were washed using RPMI 1640 and the red blood cells lysed using 1× red blood cell lysis buffer (eBioscience, Inc., San Diego, CA) and incubated for 1 min at room temperature. The cells were then washed with RPMI 1640, counted on a Cellometer Auto T4 cell counter (Nexcelom, Lawrence, MA), and resuspended in staining medium (PBS containing 0.1% NaN₃ and 1% bovine serum albumin (Sigma, Illinois)) for flow cytometry. The brain was divided into the ischemic (right) and nonischemic (left) hemispheres for analysis. The brain tissue was digested for 60 min with 1 mg/mL Type IV collagenase (Sigma Aldrich, St. Louis, MO) and DNase I (50 mg/mL, Roche Diagnostics, Indianapolis, IN) at 37°C with intermittent shaking. Samples were mixed with a 1 mL pipette every 15 min. The cell suspension was washed 1× in RPMI, resuspended in 80% Percoll, overlaid with 40% Percoll, and centrifuged for 30 min at 1600 RPM. The cells at the interface of the 80% and 40% Percoll layers were then washed twice with RPMI 1640 and resuspended in staining medium for flow cytometry.

Analysis of cell populations by flow cytometry

Four-color (FITC, PE, APC, PECy5 and/or PerCP) fluorescence flow cytometry analyses were performed, determining cell phenotypes from spleen and brain. Roughly 2×10⁵ brain cells and 1×10⁶ splenocytes were washed with staining medium, blocked with anti-mouse

CD16/CD32 Mouse BD Fc Block™ (BD Biosciences, San Jose) and then incubated with varying combinations of the following monoclonal antibodies: CD11b (M1/70), CD45 (30-F11), CD3 (145-2C11), CD11c (HL-3), CD19 (1D3), CD4 (GK1.5), CD8 (53-6.7), CD122 (TM-β1), CD44 (IM7), CD69 (H1.2F3), Ly6G (RB6-8C5) and CD25 (7D4) (all purchased from BD Biosciences, San Jose, CA) for 20 min at 4°C. 7-AAD was used to identify dead cells. CD4⁺ regulatory T cells were identified using anti-Foxp3 (FJK-16s) and accompanying Fixation/Permeabilization reagents as per manufacturer's instructions (eBioscience, Inc., San Diego, CA). Isotype matched mAb served as a negative control. Data were collected with BD Accuri™ C6 software on a BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA).

Intracellular staining

Spleen cells from individual mice were cultured at 1×10^6 cells/well in a 24-well culture plate in stimulation medium (RPMI, 1% sodium pyruvate, 1% L-glutamine, 0.4% 2-β-mercaptoethanol, 2% FBS) with PMA (50 ng/mL), ionomycin (500 ng/mL) and brefeldin A (1 μL/mL) (all reagents from Sigma-Aldrich, St. Louis, MO) for 4 hours at 37°C. Cells were blocked and surface stained (as described above), then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), according to manufacturer's instructions. Fixed cells were washed with 1x permeabilization buffer (BD Biosciences) and incubated with the following antibodies: TNF-α, IL-10 or CD206. Isotype matched antibodies served as negative controls to establish background staining levels. Data were collected with BD Accuri™ C6 software on a BD Accuri™ C6 (BD Biosciences, San Jose, CA).

Real-time PCR

Ischemic hemispheres were harvested from mice 96 hours after MCAO, flash frozen in liquid nitrogen and stored at -80°C until processed. Spleens were processed as described above and 1×10^6 cells were flash frozen in liquid nitrogen and stored at -80°C until processed. Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions. (Qiagen, Valencia, CA). cDNA was synthesized using the SuperScript II Reverse Transcriptase cDNA synthesis kit (Life Technologies, Grand Island, NY). Quantitative real-time PCR was performed using the StepOnePlus Real-Time PCR System with TaqMan primers for mouse immune response (Life Technologies, Grand Island, NY). Primers used are as follows: IL-6 (Mm00446190_m1), TNFα (Mm00443258_m1), IL-1β (Mm00434228_m1), iNOS (Mm00440502_m1), MMP-9 (Mm00442991_m1), CD206 (Mm01329362_m1), ICAM-1 (Mm00516023_m1), VCAM-1 (Mm01320970_m1), p-Selectin (Mm01295931_m1). GAPDH housekeeping gene was used as an endogenous control. Results were analyzed using ExpressionSuite Software (Thermo Fisher Scientific, Waltham, MA).

Statistical Analysis

The data were assessed by the Student's t-test using Prism (GraphPad Software, La Jolla, CA) and presented as the means ± standard error of the mean (SEM). $P < 0.05$ was considered a statistically significant difference.

Results

Male and female PPAR α KO mice were subjected to MCAO for 60 minutes. Infarct volumes were measured 96 hours after reperfusion. Male PPAR α KO mice had significantly larger infarcts compared to female PPAR α KO mice after MCAO (Fig. 1a). Similarly, male PPAR α KO mice exhibited significantly greater neurological deficit scores than female PPAR α KO mice 96 hours after experimental stroke (Fig. 1b).

Ischemic hemispheres were also harvested 96 hours after reperfusion and analyzed for activated and infiltrated immune cells. There were no significant differences between PPAR α KO male and female mice in total mononuclear cell number, frequency of immune subsets, or cell death (data not shown). Next we examined gene expression at the mRNA level in the ischemic hemisphere. We compared PPAR α KO sham to PPAR α KO MCAO mice, WT MCAO to PPAR α KO MCAO mice, and finally PPAR α KO male MCAO to PPAR α KO female mice. PPAR α KO MCAO mice exhibited an overall increase in gene expression at the mRNA level compared to sham mice for both males and females (Fig. 2). Specifically, we observed a significant increase of expression in IL-1 β , IL-6, TNF α , MMP9, ICAM-1 and p-selectin genes and a trend increase in the CD206 gene with PPAR α KO MCAO male mice compared to PPAR α KO sham male mice (Fig. 2a). Female PPAR α KO MCAO mice showed a significant increase in TNF α , MMP9, ICAM-1, VCAM-1 and p-selectin genes and a trend increase in IL-1 β and IL-6 genes compared to PPAR α KO sham female mice (Fig. 2b). When we compared male and female PPAR α KO mice, the sham group showed some slight changes in baseline sex differences. There was a slight but significant decrease in VCAM-1 gene expression in the female PPAR α KO brain compared to males and an increase in p-selectin gene expression (Fig. 3a). There were no significant sex differences in PPAR α KO mice 96 hours after MCAO (Fig. 3b).

Since PPAR α is highly expressed in peripheral lymphoid organs and their resident immune cells, we examined the immune response in the spleen after sham surgery or MCAO in PPAR α KO mice. Male and female PPAR α KO MCAO mice had significantly less splenocytes and lost sex difference in number of splenocytes compared to sham surgery PPAR α KO mice (Figure 4a). Female PPAR α KO MCAO mice had a significantly greater frequency of CD3 $^{+}$ T cells, which can be attributed to an increase in the CD4 $^{+}$ but not the CD8 $^{+}$ subset, compared to sham female PPAR α KO mice (Fig. 4b,c,d). PPAR α KO females had a greater baseline frequency of CD11b $^{+}$ cells after sham surgery compared to males, which, unlike males, significantly drops after MCAO (Fig. 4e). There were no significant differences in the frequency of CD11c $^{+}$ between PPAR α KO sham or MCAO mice or between sexes (Fig. 4f). Male PPAR α KO sham mice had a significantly greater frequency of splenic B cells compared to sham females but not MCAO male mice, while PPAR α KO females significantly decrease the frequency of B cells after MCAO compared to sham surgery (Fig. 4g).

We next examined the cell specific intracellular cytokine production and regulatory phenotype of splenocytes after either sham or MCAO surgery in male and female PPAR α KO mice. Our results indicated that production of the pro-inflammatory cytokine TNF α , produced from either CD3 $^{+}$ T cells or CD11b $^{+}$ monocytes/macrophages, was more frequent

in female sham compared to male sham PPAR α KO mice (Fig. 5a,b). The sex difference in TNF α production was lost after MCAO with CD11b+ cells but not CD3+ T cells (Fig. 5a,b). Male PPAR α KO MCAO mice exhibited a significant increase in CD11b+CD206+ macrophage M2 phenotype over female MCAO mice and that difference was not observed in sham animals (Fig. 5c). Female PPAR α KO sham mice had a greater frequency of CD4+Foxp3+ regulatory T cells (Tregs) than male PPAR α KO sham mice, and that sex difference was lost after MCAO (Fig. 5d). Interestingly, the frequency of CD8+ Tregs (CD8+CD122+) followed the same trends as CD4+ Tregs; sex difference was only observed in the sham PPAR α KO mice, yet the IL-10 production by those CD8+CD122+ cells was significantly greater in PPAR α KO MCAO males compared to PPAR α KO MCAO females (Fig. 5e,f).

Finally, we looked at expression of various immune or inflammation related genes in splenocytes of PPAR α KO male and female mice after sham surgery or MCAO. Compared to sham surgery, male PPAR α KO that have had MCAO exhibited an increase in gene expression at the mRNA level in the majority of genes examined (Fig. 6a). The increase in expression at the mRNA level was significant with IL-1 β , TNF α , CD206, ICAM-1 and p-selectin genes (Fig. 6a). On the contrary, overall gene expression at the mRNA level from splenocytes in female PPAR α KO MCAO mice went down compared to sham females (Fig. 6b). There was a significant or trend decrease in TNF α , iNOS, ICAM-1 and p-selectin gene expression with one exception to the trend being the IFN γ gene (Fig. 6b). When we compared male and female PPAR α KO sham or MCAO mice, we again saw overall trends in splenocyte gene expression at the mRNA level. In PPAR α KO sham surgery mice, females overwhelmingly exhibited greater splenocyte expression at the mRNA level with significance in IL-1 β , CD206, iNOS, ICAM-1 and p-selectin genes (Fig. 7a). In contrast, male PPAR α KO mice that have undergone MCAO surgery exhibited overall greater splenocyte gene expression at the mRNA level compared to female MCAO mice (Fig. 7a). There was a significant or nearly significant decrease in expression of TNF α , CD206, iNOS, ICAM-1 and p-selection genes in female PPAR α KO MCAO mice compared to male PPAR α KO MCAO mice (Fig. 7b).

Discussion

PPAR α has previously been implicated as a regulatory factor in the immune response. In fact, numerous studies have indicated that activation of PPAR α improves stroke outcome in males through immune regulatory mechanisms. Specifically, activation of PPAR α improves stroke outcomes by increasing cerebral blood flow after reperfusion (Guo et al. 2010). PPAR α activation reduces the expression of adhesion molecules and decreases adherent leukocytes and infiltrating neutrophils during stroke. Activation of PPAR α also improves stroke outcome by regulating oxidative stress in the brain (Collino et al. 2006; Deplanque et al. 2003; Ouk et al. 2014a) and by reducing expression of CXCL10, CXCL1 and SAA-1 genes in the liver (Losey et al. 2015). However, our group recently demonstrated that PPAR α activation based protection did not extend to females (Dotson et al. 2015a).

The difference in stroke protection through PPAR α activation is likely due to the lower levels of PPAR α expression on immune cells in females. We previously observed a lower

expression of the PPAR α gene at the mRNA level in the ischemic hemisphere in females compared to males (Dotson et al. 2015a). Additionally, it was shown that males express more PPAR α in the liver and in T cells than females and have different monocyte expression profiles (Dunn et al. 2007; Jalouli et al. 2003; Wege et al. 2015). PPAR α activation or deletion resulted in different outcomes between the sexes in many disease models. Males that do not express PPAR α develop more severe clinical signs of experimental autoimmune encephalomyelitis (EAE) suggesting that sex differences in Th1 based autoimmunity could be due, in part, to PPAR α expression in T cells of males (Dunn et al. 2007). When assessing treatments for bile acid toxicity in cholestatic liver diseases, PPAR α activation was shown to significantly reduce total bile acid concentration in men but not in women (Trottier et al. 2011). Additionally, men with type 2 diabetes responded to combination therapy that included PPAR α activation, whereas women did not respond (Ginsberg et al. 2010).

In the current study, we saw that knocking out PPAR α preserved sex differences in stroke outcome which was largely based in the peripheral immune response. Loss of PPAR α perpetuated the ischemic sex difference previously reported in WT mice (Banerjee et al. 2013; Dotson et al. 2015b), illustrated by significantly larger infarct volumes in PPAR α KO males compared to females. MCAO PPAR α KO mice had an overall increase in ischemic hemisphere gene expression at the mRNA level compared to sham surgery mice of the same sex. However, there were few baseline differences in brain gene expression at the mRNA level between the sexes of PPAR α KO sham mice and no sex differences after MCAO. The same lack of sex differences was observed in frequency of immune cell subsets in the brain after MCAO.

This result led us to examine the immune response in the spleen as representation of the peripheral immune response. Although PPAR α is expressed in specific regions of the brain, it is not the predominant isoform in the brain and is largely expressed in the periphery (Braissant et al. 1996; Moreno et al. 2004). We observed shifts in total number of splenocytes and the frequency of immune cell subsets between sham and MCAO PPAR α KO mice, but no major sex differences were seen in immune subset frequencies of PPAR α KO mice after MCAO. There was a significant increase in CD11b+CD206+ and IL-10 producing CD8+ Tregs in male PPAR α KO mice after MCAO compared to females. An increase in regulatory cells is observed with MCAO (Offner et al. 2006b); therefore it is possible that male PPAR α KO mice exhibit an increase in M2 macrophages and CD8+ Tregs in response to a worse stroke outcome than females. Interestingly, sham PPAR α KO females exhibited a baseline increase in inflammation and regulatory cells compare to males which was largely lost after MCAO, with the exception of TNF α producing T cells.

When we compared expression of inflammatory or immunoregulatory genes in splenocytes there were some notable trends. As expected, male PPAR α KO mice that had MCAO surgery consistently exhibited greater overall gene expression in the spleen at the mRNA level compared to sham surgery PPAR α KO male mice. However, female PPAR α KO MCAO mice largely had a decrease in overall gene expression at the mRNA level compared to sham mice with the exception of the IFN γ gene. Additionally, female sham PPAR α KO mice displayed greater overall gene expression at the mRNA level in the spleen compared to

sham PPAR α KO males while male MCAO PPAR α KO mice mostly displayed greater peripheral gene expression at the mRNA level than MCAO PPAR α KO females.

These data suggest that the loss of PPAR α initiate a baseline immune cell resting state in males but not females and at 96 hours after experimental stroke, males shift to an activated state while females maintain a less inflammatory phenotype. The data also illustrate that the peripheral immune response is predominately responsible for sex differences after experimental stroke in PPAR α KO mice. Recent studies report that higher expression of PPAR α in male mice correlated with elevated Th2 cytokine production of these cells, thus preponderance of autoreactivity in females (Dunn et al. 2007). In fact, in a previous study, our group hypothesized that lower expression of PPAR α in females could be responsible for a worse long-term stroke outcome observed in females (Dotson et al. 2015a). Loss of PPAR α also shifted the Th1/Th2 balance to the pro-inflammatory Th1 phenotype (Yessoufou et al. 2006). The shift to a Th1 phenotype could hypothetically have greater consequences for males which rely partially on PPAR α for its inflammatory regulating Th2 phenotype, hence, a poorer outcome when PPAR α is knocked out. It is also plausible that sex hormones play a role in PPAR α sex differences, as there is a suggested interaction between PPAR α and sex hormones. PPAR α has been shown to be involved in estrogen signaling and PPAR α expression can be altered with testosterone or estrogen treatment (Jalouli et al. 2003; Leuenberger et al. 2009).

Taken together, this study illustrates that, similar to previous studies, loss of PPAR α in inflammatory diseases leads to sex difference in disease outcome. In this case, loss of PPAR α perpetuates sex difference in stroke outcome. That sex difference is driven by the peripheral immune response, specifically by an increase in pro-inflammatory and adhesion molecule gene expression at the mRNA level in males after MCAO. Our data supports previous claims that PPAR α is partially responsible for sex differences in the immune response.

Acknowledgments

The authors wish to thank Gail Kent for assistance with manuscript submission. This work was supported by NIH/NINDS 5R01NS076013 (HO, JAS). This material is based upon work supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development. The contents do not represent the views of the Department of Veterans Affairs or the United States Government.

Abbreviations

PPARα	Peroxisome proliferator-activated receptor alpha
MCAO	middle cerebral artery occlusion
Treg	regulatory T cell

References

- Banerjee A, Wang J, Bodhankar S, Vandenbark AA, Murphy SJ, Offner H. Phenotypic changes in immune cell subsets reflect increased infarct volume in male vs. female mice. *Transl Stroke Res*. 2013; 4:554–563.10.1007/s12975-013-0268-z [PubMed: 24187596]

- Bishop-Bailey D. Peroxisome proliferator-activated receptors in the cardiovascular system. *British journal of pharmacology*. 2000; 129:823–834.10.1038/sj.bjp.0703149 [PubMed: 10696077]
- Braissant O, Fougelle F, Scotto C, Dauca M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology*. 1996; 137:354–366.10.1210/endo.137.1.8536636 [PubMed: 8536636]
- Chinetti G, et al. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. *The Journal of biological chemistry*. 1998; 273:25573–25580. [PubMed: 9748221]
- Collino M, et al. Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: effects of the PPAR-alpha agonist WY14643. *Free radical biology & medicine*. 2006; 41:579–589.10.1016/j.freeradbiomed.2006.04.030 [PubMed: 16863991]
- Cullingford TE, Bhakoo K, Peuchen S, Dolphin CT, Patel R, Clark JB. Distribution of mRNAs encoding the peroxisome proliferator-activated receptor alpha, beta, and gamma and the retinoid X receptor alpha, beta, and gamma in rat central nervous system. *Journal of neurochemistry*. 1998; 70:1366–1375. [PubMed: 9523552]
- Delerive P, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors in inflammation control. *The Journal of endocrinology*. 2001; 169:453–459. [PubMed: 11375115]
- Deplanque D, et al. Peroxisome proliferator-activated receptor-alpha activation as a mechanism of preventive neuroprotection induced by chronic fenofibrate treatment. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2003; 23:6264–6271. [PubMed: 12867511]
- Di Carlo A, et al. Sex differences in the clinical presentation, resource use, and 3-month outcome of acute stroke in Europe: data from a multicenter multinational hospital-based registry. *Stroke*. 2003; 34:1114–1119.10.1161/01.str.0000068410.07397.d7 [PubMed: 12690218]
- Dotson AL, Wang J, Chen Y, Manning D, Nguyen H, Saugstad JA, Offner H. Sex differences and the role of PPAR alpha in experimental stroke. *Metabolic brain disease*. 2015a10.1007/s11011-015-9766-x
- Dotson AL, Wang J, Saugstad J, Murphy SJ, Offner H. Splenectomy reduces infarct volume and neuroinflammation in male but not female mice in experimental stroke. *J Neuroimmunol*. 2015b; 278:289–298.10.1016/j.jneuroim.2014.11.020 [PubMed: 25434281]
- Dunn SE, et al. Peroxisome proliferator-activated receptor (PPAR)alpha expression in T cells mediates gender differences in development of T cell-mediated autoimmunity. *The Journal of experimental medicine*. 2007; 204:321–330.10.1084/jem.20061839 [PubMed: 17261635]
- Ginsberg HN, et al. Effects of combination lipid therapy in type 2 diabetes mellitus. *The New England journal of medicine*. 2010; 362:1563–1574.10.1056/NEJMoa1001282 [PubMed: 20228404]
- Guo Q, Wang G, Namura S. Fenofibrate improves cerebral blood flow after middle cerebral artery occlusion in mice. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2010; 30:70–78.10.1038/jcbfm.2009.185
- Hurn PD, Subramanian S, Parker SM, Afentoulis ME, Kaler LJ, Vandenbark AA, Offner H. T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2007; 27:1798–1805.10.1038/sj.jcbfm.9600482
- Jalouli M, et al. Sex difference in hepatic peroxisome proliferator-activated receptor alpha expression: influence of pituitary and gonadal hormones. *Endocrinology*. 2003; 144:101–109.10.1210/en.2002-220630 [PubMed: 12488335]
- Jones DC, Ding X, Daynes RA. Nuclear receptor peroxisome proliferator-activated receptor alpha (PPARalpha) is expressed in resting murine lymphocytes. The PPARalpha in T and B lymphocytes is both transactivation and transrepression competent. *The Journal of biological chemistry*. 2002; 277:6838–6845.10.1074/jbc.M106908200 [PubMed: 11726654]
- Kainu T, Wikstrom AC, Gustafsson JA, Peltö-Huikko M. Localization of the peroxisome proliferator-activated receptor in the brain. *Neuroreport*. 1994; 5:2481–2485. [PubMed: 7696585]
- Leuenberger N, Pradervand S, Wahli W. Sumoylated PPARalpha mediates sex-specific gene repression and protects the liver from estrogen-induced toxicity in mice. *The Journal of clinical investigation*. 2009; 119:3138–3148.10.1172/jci39019 [PubMed: 19729835]

- Losey P, Ladds E, Laprais M, Geuvel B, Burns L, Bordet R, Anthony DC. The role of PPAR activation during the systemic response to brain injury. *Journal of neuroinflammation*. 2015; 12:99.10.1186/s12974-015-0295-7 [PubMed: 25994490]
- Marx N, Sukhova GK, Collins T, Libby P, Plutzky J. PPARalpha activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells. *Circulation*. 1999; 99:3125–3131.
- Moreno S, Farioli-Vecchioli S, Ceru MP. Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat. *CNS Neuroscience*. 2004; 123:131–145. [PubMed: 14667448]
- Mozaffarian D, et al. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. *Circulation*. 2015; 131:e29–322.10.1161/cir.000000000000152 [PubMed: 25520374]
- Offner H, Subramanian S, Parker SM, Afentoulis ME, Vandenbark AA, Hurn PD. Experimental stroke induces massive, rapid activation of the peripheral immune system. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2006a; 26:654–665.10.1038/sj.jcbfm.9600217
- Offner H, et al. Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. *Journal of immunology (Baltimore, Md : 1950)*. 2006b; 176:6523–6531.
- Ouk T, et al. Effects of the PPAR-alpha agonist fenofibrate on acute and short-term consequences of brain ischemia. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2014a; 34:542–551.10.1038/jcbfm.2013.233
- Poynter ME, Daynes RA. Peroxisome proliferator-activated receptor alpha activation modulates cellular redox status, represses nuclear factor-kappaB signaling, and reduces inflammatory cytokine production in aging. *The Journal of biological chemistry*. 1998; 273:32833–32841. [PubMed: 9830030]
- Seifert HA, Hall AA, Chapman CB, Collier LA, Willing AE, Pennypacker KR. A transient decrease in spleen size following stroke corresponds to splenocyte release into systemic circulation. *J Neuroimmune Pharmacol*. 2012a; 7:1017–1024.10.1007/s11481-012-9406-8 [PubMed: 23054371]
- Seifert HA, et al. The spleen contributes to stroke induced neurodegeneration through interferon gamma signaling. *Metabolic brain disease*. 2012b; 27:131–141.10.1007/s11011-012-9283-0 [PubMed: 22354752]
- Trottier J, Caron P, Straka RJ, Barbier O. Profile of serum bile acids in noncholestatic volunteers: gender-related differences in response to fenofibrate. *Clinical pharmacology and therapeutics*. 2011; 90:279–286.10.1038/clpt.2011.124 [PubMed: 21716269]
- Turner RC, Lucke-Wold B, Lucke-Wold N, Elliott AS, Logsdon AF, Rosen CL, Huber JD. Neuroprotection for ischemic stroke: moving past shortcomings and identifying promising directions. *Int J Mol Sci*. 2013; 14:1890–1917.10.3390/ijms14011890 [PubMed: 23344061]
- Wege N, et al. Men and women differ in their diurnal expression of monocyte peroxisome proliferator-activated receptor-alpha in the fed but not in the fasted state. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2015; 29:2905–2911.10.1096/fj.14-267575 [PubMed: 25825462]
- Yessoufou A, Hichami A, Besnard P, Moutairou K, Khan NA. Peroxisome proliferator-activated receptor alpha deficiency increases the risk of maternal abortion and neonatal mortality in murine pregnancy with or without diabetes mellitus: Modulation of T cell differentiation. *Endocrinology*. 2006; 147:4410–4418.10.1210/en.2006-0067 [PubMed: 16763063]
- Zhang W, et al. Soluble epoxide hydrolase gene deletion is protective against experimental cerebral ischemia. *Stroke*. 2008; 39:2073–2078.10.1161/strokeaha.107.508325 [PubMed: 18369166]

Figure 1.

Loss of PPAR α does not resolve sex differences in infarct volume after MCAO. Male and female mice were subjected to transient MCAO (60 min). Brains were harvested 96 h after MCAO and brain slices were stained with 2,3,5- TTC. Infarct volumes of male and female mice were measured as percentage of contralateral structure (a). Neurological deficit score was also measured 96 hours after MCAO (b). Values represent mean numbers ($\pm$ SEM). n=6 for male and 6 female mice. * indicates p<0.05. ** indicates p<0.01.

Figure 2.

Comparison of ischemic gene expression between sham surgery and MCAO in PPAR α KO mice. Brains were harvested 96 hours after sham surgery or MCAO. mRNA was isolated from the ischemic hemispheres and analyzed by real-time PCR. Brain tissue from male (**a**) and female (**b**) mice was examined and results are relative to the sham group which was normalized to 1. RE= relative gene expression. Values represent mean numbers ($\pm$ SEM) of 3 mice per group. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ by t-test.

Figure 3.

Comparison of ischemic gene expression between male and female PPAR α KO after sham surgery or MCAO. Brains were harvested 96 hours after sham surgery or MCAO. mRNA was isolated from the ischemic hemispheres and analyzed by real-time PCR. Brain tissue from sham (**a**) and MCAO (**b**) mice was examined and results are relative to the male group which was normalized to 1. RE= relative gene expression. Values represent mean numbers ($\pm$ SEM) of 3 mice per group. * indicates $p < 0.05$ and ** indicates $p < 0.01$ by t-test.

Figure 4.

Immune subsets in the spleen after MCAO. Spleens were harvested 96 hours after sham surgery or MCAO and splenocytes were immunophenotyped by flow cytometry in male and female mice. Total splenocytes were enumerated; n=4 male sham, 4 female sham, 11 male MCAO, 10 female MCAO (a). The frequency of CD3+ T cells, CD4+ T cells, CD8+ T cells, CD11b+ myeloid cells, CD11c+ dendritic cells and CD19+ B cells were determined (b–g). Values represent mean numbers (±SEM) of n=4 male sham, 4 female sham, 5 male MCAO, 4 female MCAO. * indicates p<0.05 and ** indicates p <0.01 by t-test.

Figure 5.

Effector and regulatory cells in the spleen after MCAO. Spleens were harvested 96 hours after MCAO and splenocytes were immunophenotyped by flow cytometry in male and female mice after sham surgery or MCAO. TNF α production was determined by gating on CD3+ or CD11b+ subsets and measuring TNF α positive cells compared to isotype (**a**, **b**). CD206 expression was determined by gating on CD11b+ cells and measuring CD206 positive cells compared to isotype (**c**). The frequency of CD4+ regulatory T cells was determined by gating on CD4+ cells before measuring Foxp3 positive cells (**d**). The frequency of CD8+ regulatory T cells was determined by gating on CD3+ and CD8+ cells before measuring CD122 and IL-10 expression (**e**, **f**). Values represent mean numbers ($\pm$ SEM) of 4 mice per group. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ by t-test.

Figure 6.

Comparison of gene expression in the spleen between sham surgery and MCAO in PPARα KO mice. Spleens were harvested 96 hours after sham surgery or MCAO. mRNA was isolated from the ischemic hemispheres and analyzed by real-time PCR. Splenocytes from male (a) and female (b) mice were examined and results are relative to the sham group which was normalized to 1. RE= relative gene expression. Values represent mean numbers (±SEM) of 3 mice per group. * indicates $p < 0.05$ and ** indicates $p < 0.01$ by t-test.

Figure 7.

Comparison of gene expression in the spleen between male and female PPAR α KO after sham surgery or MCAO. Spleens were harvested 96 hours after sham surgery or MCAO. mRNA was isolated from the ischemic hemispheres and analyzed by real-time PCR. Splenocytes from sham (**a**) and MCAO (**b**) mice were examined and results are relative to the male group which was normalized to 1. RE= relative gene expression. Values represent mean numbers ($\pm$ SEM) of 3 mice per group. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ by t-test.