Published in final edited form as:

Metab Brain Dis. 2016 June ; 31(3): 539–547. doi:10.1007/s11011-015-9766-x.

Sex differences and the role of PPAR alpha in experimental stroke

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Abstract

Males and females respond differently to stroke. Moreover, females often experience worse long-term stroke outcomes. Fenofibrate, a peroxisome proliferator-activated receptor alpha (PPAR α) agonist has been shown to improve stroke outcome and resolve neuroinflammation in male mice. The present study compares the effect of pretreatment with fenofibrate versus vehicle control in male and female mice during experimental stroke. Mice were treated with low-dose fenofibrate 30 minutes before and once a day for three additional days after stroke onset. We observed a reduction in infarct volume in male mice 96 hours post-stroke with low-dose fenofibrate pretreatment that was due to increase of an M2 macrophage phenotype in the brain and an increase in regulatory cells in the periphery. These outcomes were not replicated in females, likely due to the lower PPAR α expression in cells and tissues in females vs males. We conclude that PPAR α agonist treatment prior to stroke is neuroprotective in males but not females. These findings indicate PPAR α as a probable mechanism of sex difference in stroke outcome and support the need for representation of females in stroke therapy research.

Keywords

Experimental stroke; Sex difference; Inflammation; PPAR α ; fenofibrate

Introduction

Stroke is a leading cause of death and disability in the United States (Mozaffarian et al. 2015) and the immune response plays an important role in stroke outcome. Following

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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.

ischemic injury, peripheral leukocytes become activated and can infiltrate ischemic tissue where they perpetuate neurodegeneration and increase infarct volume (Offner et al. 2006a; Offner et al. 2006b; Seifert et al. 2012a; Seifert et al. 2012b). The immune response to stroke differs between males and females. For instance, male mice exhibit an initial increase in infarction characterized by an early and more robust recruitment of CD45^{high}CD11b⁺ macrophages into post-ischemic brain relative to females (Banerjee et al. 2013; Dotson et al. 2015). If the spleen is removed prior to MCAO, sex differences in infarct and activated microglia/macrophages in the brain is lost due to, in part, a decrease of peripheral CD11b⁺ cells in males only (Dotson et al. 2015). Despite a poor initial stroke outcome in males, females often suffer from more long-term stroke effects leading to higher incidence of disability and a substandard quality of life (Di Carlo et al. 2003).

The difference in how males and females respond to stroke needs to be considered when defining successful stroke therapies. Hundreds of potential stroke therapies and neuroprotectants that have been successful in preclinical studies failed to be translated to successful stroke therapies in humans (Turner et al. 2013). A major contributing factor to the lack of clinical success in stroke therapy is the underrepresentation of females in therapy-based basic research.

A promising target for stroke therapy is peroxisome proliferator-activated receptor alpha (PPAR α). PPAR α is a nuclear hormone receptor and ligand-activated transcription factor that regulates lipid metabolism in the liver. PPAR α also negatively regulates inflammation. PPAR α ligands inhibit pro-inflammatory cytokines by interfering with the NF- κ B signaling pathway (Delerive et al. 2001). In addition to inflammation, PPAR α plays a role in oxidative stress and leukocyte-endothelial interactions (Marx et al. 1999; Poynter and Daynes 1998). PPAR α is expressed in brain tissue such as neurons and astrocytes, cerebral vascular endothelial and in monocytes/macrophages (Bishop-Bailey 2000; Cullingford et al. 1998; Kainu et al. 1994). In the periphery, PPAR α is expressed in the liver, the intestines, specifically the Peyer's patch, and in the white pulp of the spleen (Braissant et al. 1996; Moreno et al. 2004). At a cellular level, PPAR α is expressed in macrophages, T cells and B cells (Chinetti et al. 1998; Jones et al. 2002). Together, the regulation of inflammation, oxidative stress and leukocyte extravasation along with cerebral and peripheral expression has made PPAR α an attractive target for therapy of ischemia.

PPAR α agonist treatment has been effective at reducing infarction in males when given prior to stroke onset (Ouk et al. 2013). Specifically, PPAR α agonist therapy before stroke reduces adhesion molecule expression in the brain thereby inhibiting early neutrophil infiltration and consequential microglia activation (Ouk et al. 2013). However, the effect of PPAR α agonists on stroke has yet to be studied in females. Males and females differ in expression of PPAR α receptors. In liver, PPAR α mRNA and protein are expressed at higher level in the male and mediate several sex differences in metabolism (Jalouli et al. 2003). PPAR α mRNA and protein are also expressed at higher basal levels in male vs. female lymphocytes (Dunn et al. 2007). Further, PPAR α deficiency selectively alters male, but not female, T lymphocyte function and immune virulence (Dunn et al. 2007). Therefore, it is imperative that we examine how females respond to PPAR α agonist stroke therapy.

In the present study, we compare PPAR α agonist therapy during stroke to vehicle control in male and female mice. Mice were treated via intraperitoneal (i.p.) injection with low-dose fenofibrate 30 minutes prior to transient MCAO, and then each day after until brain and spleens were harvested at 96 hours post-stroke. We found that males, but not females, showed improved infarct size 96 hours after stroke onset with low-dose fenofibrate. Males had an increase in M2 macrophage gene expression in ischemic tissue when treated with fenofibrate while females exhibited an increase in pro-inflammatory cytokine gene expression. Finally, while fenofibrate decreased inflammatory cytokine production in peripheral immune cells after stroke of both males and females, the frequency of regulatory cells in the spleen was increased in males but decreased in female mice. These data indicate that males and females respond to PPAR α activation differently and perpetuate the crucial need for representation of females in stroke therapy research.

Materials and Methods

Ethics Statement

The study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the Portland Veteran Affairs Medical Center Institutional Animal Care and Use Committee, protocol # 2840-12, 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 (The Jackson Laboratory, Sacramento, CA, USA) weighing 20-25 g were housed in a climate-controlled room on a 12-hour light/dark cycle. Food and water were provided ad libitum.

Middle Cerebral Artery Occlusion Model

Transient focal cerebral ischemia was induced in male and female mice for 1 hour by 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.

Fenofibrate treatment

Mice were randomized to receive 0.2mL (300µg) fenofibrate or 0.2mL vehicle (10% DMSO + 2% tween 80 in sterile saline) by i.p. injection 30 minutes after stroke surgery and once a day for three additional days. Mice were euthanized at 96 hours after stroke onset.

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 noninfarcted 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.

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 were then washed twice with RPMI 1640 and resuspended in staining medium for flow cytometry.

Analysis of cell populations by flow cytometry

Antibodies were purchased (BD Biosciences, San Jose, CA or eBioscience, Inc., San Diego, CA). Four-color (FITC, PE, APC, PECy5 and/or PerCP) fluorescence flow cytometry analyses were performed, determining cell phenotypes from spleen and brain. Roughly 2×10^5 brain cells and 1×10^6 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 (MAC-1), CD45 (Ly-5), 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) 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 (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 μ L/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 and were harvested from mice 96 hours after MCAO, frozen in liquid nitrogen and stored at -80°C until processed. Spleens were processed as described above and 1×10^6 cells were 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 realtime 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), TNF α (Mm00443258_m1), IL-1 β (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 (Life Technologies, Grand Island, NY).

Statistical Analysis

Data are presented as mean $\pm$ SEM. Statistical comparisons were made between vehicle and fenofibrate for infarct, gene expression and cellular subtype. Statistical differences were performed with the Student's t-test using Prism (GraphPad Software, La Jolla, CA). Statistical significance was $p < 0.05$.

Results

Numerous studies have reported resolution of infarct volume and neuroinflammatory factors with PPAR α agonist pretreatment in males (Ouk et al. 2013). However, the response that female mice have to PPAR α agonist therapy during stroke has yet to be determined. Thus we examine the response to PPAR α agonist therapy during stroke in female mice. Additionally, we examined the effects of a lower dose of fenofibrate than previously tested in males and

females. Male and female mice received 300ug (about 15mg/kg) of fenofibrate 30 minutes prior to MCAO and then again each day until 96 hours post-reperfusion when infarct volume was examined. Even with a lower concentration of fenofibrate, male mice were protected with PPAR α agonist pretreatment, exhibiting significantly smaller infarct volumes in the cortex, striatum and hemisphere (Fig. 1a). Female mice, on the other hand, showed no change in infarct volumes (Fig. 1b). These results indicate that low-dose PPAR α agonist therapy protects males, but not females, during ischemic stroke injury.

Next, we looked at the frequency of leukocytes in the ischemic hemisphere to determine if leukocyte infiltration was effected by PPAR α agonist therapy. CD11b+CD45+ cells are activated microglia/infiltrating monocytes and CD11b+Ly6G+ cells are infiltrating neutrophils. Both cell types perpetuate inflammatory conditions after ischemia. We did not observe any significant differences in CD11b+CD45+ or CD11b+Ly6G+ frequency between vehicle and fenofibrate in either male (Fig. 2a) or female (Fig. 2b) mice. CD19+ B cells can be potentially beneficial for stroke outcome (Bodhankar et al. 2014). Again, there were no significant differences in B cell frequency with vehicle vs. fenofibrate treated male or female mice in the ischemic hemisphere (Fig. 2a and 2b, respectively). There was, however, a significant decrease in the frequency of 7-aminoactinomycin D (7AAD+) staining of dead cells with fenofibrate in both male (Fig. 2a) and female (Fig. 2b) mice, indicating that PPAR α agonist therapy reduces cell death after ischemia.

We also examined pro- and anti-inflammatory gene expression in the ischemic hemisphere after PPAR α agonist therapy. There were no significant differences in expression of the pro-inflammatory cytokines IL-6, TNF α and IL-1 β in the ischemic tissue between vehicle and fenofibrate with males (Fig. 3a). However, females treated with fenofibrate during experimental stroke significantly upregulated expression of TNF α and exhibited a trend upregulation of IL-6 in ischemic tissue compared to their vehicle controls (Fig 3b). There was no difference in the ischemic tissue gene expression of inducible nitric oxide synthase (iNOS) in either males or females (Fig. 3a and 3b, respectively). As opposed to females, males treated with fenofibrate during stroke show a significant increase in matrix metalloproteinase 9 (MMP-9) and M2 macrophage marker, CD206 (Fig. 3a). Neither males nor females exhibited any significant differences in ischemic tissue with adhesion molecules (ICAM-1 and VCAM-1) or p-selectin between vehicle and PPAR α agonist treatment during experimental stroke.

Additionally, we compared PPAR α expression in the ischemic hemisphere 96 hours after experimental stroke between males and females. We found a trend decrease in PPAR α expression in vehicle female mice compared to males after MCAO (Fig. 4a). Moreover, there was a significant decrease in PPAR α expression in female compared to male ischemic tissue 96 hours after stroke (Fig. 4b) suggesting a lower baseline expression of PPAR α expression in females after stroke that is perpetuated with PPAR α agonist. These data also suggest a mechanism of differential response to fenofibrate.

To get an idea of how peripheral immune responses could be affecting brain-specific outcomes after stroke, we studied the effect that PPAR α agonist had on cells in the spleen. Overall, we observed no significant differences between PPAR α agonist and vehicle control

with either males or females in the frequency of T cells, monocytes/macrophages and B cells in the spleen (Table 1). There was a significant increase in dendritic cells in males treated with fenofibrate compared to vehicle control after stroke. We observed a trend decrease in CD4+CD69+ early activated cells in the spleen of males treated with fenofibrate (Table 1) but again, there were no major differences between treatment or control in activated (CD69+) or effector (CD44+) T cells in the spleens of males and females after experimental stroke (Table 1).

Although there were no major differences in immune subset frequencies in the spleen after stroke and PPAR α agonist therapy, we checked whether the effector activity of those cells was altered by treatment. Both sexes exhibited a trend of significant decrease in TNF α production by CD11b+ cells with PPAR α agonist therapy during stroke (Fig. 5a and 5b) but only males showed a sizable and significant decrease in TNF α produced by CD3+ T cells with fenofibrate treatment (Fig. 5a). PPAR α agonist did not significantly affect the frequency of CD11b+CD206 M2 macrophages in the spleen after stroke in either sex (Fig. 5a, 5b). When we examined the frequency of regulatory cells we noticed a trending increase in splenic CD122+CD8+ regulatory T cells and IL-10 produced by the CD8+ Tregs in males treated with fenofibrate during experimental stroke (Fig. 5a). Additionally, when females were given PPAR α agonist during stroke, the frequency of CD122+CD8+ Tregs and CD4+Foxp3+ Tregs were significantly or nearly significantly reduced, respectively (Fig. 5b).

Discussion

PPAR α agonist, fenofibrate, has been a promising stroke therapy in males. Fibrates are a class of amphipathic carboxylic acids that are lipid lowering drugs used to treat hypercholesterolemia and hypertriglyceridemia (Staels et al. 1998). In males, fenofibrate has been shown to improve initial stroke outcomes by reducing infarct volume and enhancing cerebral blood flow recovery after reperfusion (Guo et al. 2010). Additionally, treatment with fenofibrate before stroke reduces the expression of ICAM-1 and VCAM-1, reduces adherent leukocytes, infiltrating neutrophils and regulates oxidative stress in the brain (Deplanque et al. 2003; Ouk et al. 2014a). Pre-treatment with fenofibrate also reduces neutrophils and gene expression of CXCL10, CXCL1 and SAA-1 in the liver after stroke (Losey et al. 2015). PPAR α agonist, WY14643, exhibits a similar reduction of adhesion molecule expression, oxidative stress and iNOS expression when used as a pretreatment to stroke (Collino et al. 2006).

For the first time, we demonstrate that males are protected from stroke by low-dose fenofibrate, but females are not. Male mice had significantly smaller infarct volumes after stroke when treated with fenofibrate while females exhibited no change compared to vehicle (Fig. 1). Although other groups have observed decreases in infiltrating macrophages and neutrophils after stroke with fenofibrate treatment, we did not observe any changes in the frequency of immune subsets in males or females treated with either fenofibrate or vehicle (Fig. 2). One hypothesis for the difference compared to the literature is that we were looking at a 96 hour post-stroke time point while previous studies are looking at early times after stroke, most being 24 hours post-stroke. We also started fenofibrate treatment only 30

minutes prior to MCAO compared to many studies that pre-treat for 14 days before stroke onset. Additionally, low-dose, i.p. treatment may not exhibit the widespread effects of long-term, high-dose fenofibrate treatment. Collino, et al. did a low dose study with PPAR α agonist, WY14643, 30 minutes prior to MCAO (Collino et al. 2006). The differences they observed in oxidative stress, MAP kinase, NF- κ B, iNOS and ICAM-1 expression after stroke with and without PPAR α agonist were lost by 24 hours of reperfusion (Collino et al. 2006), suggesting that many of the PPAR α mechanisms happen early after stroke and may be completely undetectable by 96 hours.

Fenofibrate pretreatment does, however, effect gene expression in the ischemic hemisphere. Figure 3 illustrates that while males exhibit an increase in CD206 gene expression, females significantly increase TNF α expression with fenofibrate treatment prior to stroke. These data indicate that fenofibrate treatment of stroke pushes males to more of an M2 macrophage phenotype, and females an M1 macrophage phenotype. Moreover, fenofibrate caused an increase gene expression of proinflammatory cytokine, IL-6, in the ischemic hemisphere of females. It was interesting that pretreatment of stroke with fenofibrate increased MMP-9 expression in brains of male mice. Although matrix metalloproteinases are known to contribute to stroke damage by degrading the cellular matrix, they are also part of the recovery and rebuilding process days after stroke by promoting brain tissue restructuring, remodeling and movement of neurons (Lee et al. 2006; Zhao et al. 2006). Finally, we observed no difference in adhesion molecule or selectin gene expression after stroke between fenofibrate and vehicle in either sex. Again, we speculate that any differences in adhesion molecule expression would have occurred early after stroke and may be undetectable by 96 hours of reperfusion.

The effect of PPAR α agonist treatment on the peripheral immune response prior to stroke had largely been unexamined before this study. Fenofibric acid does not cross the blood-brain at a very efficient rate (Deplanque et al. 2003). Therefore, it is likely that its effects take place predominantly in the periphery. Although both males and females exhibited a decrease in TNF α secretion by immune cells in the spleen with fenofibrate treatment of stroke (Fig. 5), the sex differences were mostly observed in regulatory cell frequency. CD8 regulatory T cells express CD122 and regulate effector cell function with perforin and IL-10 (Wang and Alexander 2009). CD8 Tregs have been directly linked with a better stroke outcome after MCAO (Banerjee et al. 2013; Bodhankar et al. 2013). There are conflicting views on whether CD4 $^{+}$ regulatory T cells help or hurt stroke related neuronal damage (Schabitz 2013; Xu et al. 2013) but in general, CD4 $^{+}$ Tregs regulate the peripheral immune response using regulatory immunosuppressive cytokines (Li et al. 2013; Liesz et al. 2009; Planas and Chamorro 2009). The trending increase of CD8 Tregs in males and decrease of CD4 and CD8 Tregs in females treated with fenofibrate prior to stroke suggests that modulation of regulatory immune subsets in the periphery has important neuroinflammatory effects.

Sex differences in response to stroke are becoming better documented. For instance, the spleen and IL-4 are needed to observe sex difference in infarct size after stroke, and without either the spleen or IL-4, infarct sex difference is lost (Dotson et al. 2015; Xiong et al. 2015). Additionally, higher levels of IL-10 after stroke have been associated with poor acute and

long-term outcomes in females (Conway et al. 2015). The difference in stroke protection with fenofibrate in males but not females is likely due to the lower levels of PPAR α expression in females. We observed a lower gene expression of PPAR α in the ischemic hemisphere in females treated with fenofibrate compared to males treated with fenofibrate (Fig. 4). Males also express higher PPAR α levels in the liver and in lymphocytes than females (Dunn et al. 2007; Jalouli et al. 2003). Male but not female PPAR $\alpha^{-/-}$ knockout mice develop more severe symptoms in experimental autoimmune encephalomyelitis (EAE) suggesting that PPAR α expression in T cells of males leads them less prone to Th-1 based autoimmunity (Dunn et al. 2007). It is also likely that lower PPAR α expression in lymphocytes is partially responsible for the poor, long-term stroke outcome observed in females.

Taken together, these data illustrate that the pre-treatment of stroke with PPAR α agonist, fenofibrate, improves stroke outcome in males but not females. We observed a reduction in infarct volume in male mice 96 hours post-stroke with low-dose fenofibrate pre-treatment that was due to an increase of M2 phenotype in the brain and an increase in regulatory cells in the periphery. These findings were not replicated in females, likely due to the lower PPAR α expression in cells and tissues. In addition to the failure of PPAR α agonist pretreatment to protect against stroke in females, a previous study reports that fenofibrate does not reduce cerebral infarct volume when administered one hour after stroke onset (Deplanque et al. 2003). Therefore, it could be concluded that PPAR α agonists may not translate well to a clinical stroke therapy. Finally, this study confirms the importance for representation of females in stroke therapy research and indicates PPAR α as a probable mechanism of sex differences in stroke.

Acknowledgements

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

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Figure 1.

Fenofibrate resolves infarct volume in male but not female mice. Male and female mice were treated with fenofibrate or vehicle 30 minutes prior to, and once a day following 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 (**a**) and female (**b**) mice were measured as percentage of contralateral structure. Values represent mean numbers ($\pm$ SEM). $n=7$ for male vehicle, 10 for male fenofibrate, 7 for female vehicle and 5 for female fenofibrate. * indicates $p<0.05$. ** indicates $p<0.01$.

Figure 2.

Immune subsets in the ischemic hemisphere with fenofibrate treatment. Brains were harvested 96 hours after MCAO from vehicle or fenofibrate treated mice, processed and immunophenotyped by flow cytometry. Infiltrating and activated monocytes/microglia was determined by gating on CD11b+ cells and examining CD45 high cells. Averages of CD11b+CD45+ activated monocytes/microglia, CD11b+Ly6G+ neutrophils, CD19+ B cells and 7AAD+ dead cells of male (**a**) and female (**b**) mice are represented. Values represent mean numbers ($\pm$ SEM) of 2-4 male mice per group and 3 female mice per group.

Figure 3.

Gene expression profile from ischemic brain. Relative expression of genes in the ischemic hemisphere 96 hours after MCAO was analyzed by real-time PCR. Brain tissue from male (a) and female (b) mice was examined and results are relative to the vehicle group which was normalized to 1. Values represent mean numbers ($\pm$ SEM) of 2-3 mice per group. * indicates $p < 0.05$. ** indicates $p < 0.01$.

Figure 4.

PPAR α expression in the ischemic brain. Relative expression of PPAR α in the ischemic hemisphere 96 hours after MCAO was analyzed by real-time PCR. Brain tissue from male and female mice treated with vehicle or fenofibrate was examined. Results are relative to the male group which was normalized to 1. Values represent mean numbers ($\pm$ SEM) of 2-3 mice per group. * indicates $p < 0.05$.

Figure 5.

Effector and regulatory cells in the spleen after MCAO. Spleens were harvested 96 hours after MCAO and immunophenotyped by flow cytometry in male (a) and female (b) mice treated with or without fenofibrate. TNFα production was determined by gating on CD11b+ or CD3+ subsets and measuring TNFα positive cells compared to isotype. CD206 expression was determined by gating on CD11b+ cells and measuring CD206 positive cells compared to isotype. The frequency of CD8+ regulatory T cells was determined by gating on CD3+ and CD8+ cells before measuring CD122 and IL-10 expression. The frequency of CD4+ regulatory T cells was determined by gating on CD4+ cells before measuring Foxp3 positive cells. Values represent mean numbers (±SEM) of 4-6 mice per group for TNFα+, CD206+, CD122 and IL-10+ cells and 3-8 mice per group for CD4+ Tregs. * indicates $p < 0.05$.

Table 1

Immune subsets in the spleen 96 hours after MCAO

Splenocyte frequency	male vehicle	male fenofibrate	female vehicle	female fenofibrate
Splenocyte #	$28.6 \times 10^6 \pm 4.52 \times 10^6$	$14.6 \times 10^6 \pm 3.48 \times 10^6$ *	$31.7 \times 10^6 \pm 6.76 \times 10^6$	$17.2 \times 10^6 \pm 4.79 \times 10^6$
CD3+ (%)	39.16 ± 3.52	37.16 ± 4.35	36.55 ± 3.12	35.94 ± 6.35
CD4+ (%)	20.36 ± 1.72	20.69 ± 2.24	21.31 ± 1.75	19.34 ± 3.03
CD8+(%)	15.54 ± 1.73	14.32 ± 2.11	15.65 ± 2.15	14.55 ± 2.56
CD4+CD69+ (%)	18.07 ± 2.087	12.76 ± 1.131 ^a	17.39 ± 1.74	17.38 ± 1.83
CD8+CD69+ (%)	5.73 ± 0.54	6.83 ± 1.06	9.71 ± 1.7	5.86 ± 0.61
CD4+CD44+ (%)	27.49 ± 2.13	24.93 ± 1.46	25.18 ± 2.55	25.68 ± 2.43
CD8+CD44+ (%)	16.51 ± 2.31	23.2 ± 2.21	18.34 ± 2.1	16.35 ± 2.72
CD11b+ (%)	11.76 ± 2.89	6.88 ± 1.46	5.92 ± 0.65	7.37 ± 0.81
CD11c+ (%)	0.88 ± 0.2	1.73 ± 0.14 **	1.01 ± 0.07	0.74 ± 0.13
CD19+ (%)	37.25 ± 5.43	46.0 ± 3.8	48.34 ± 2.69	47.32 ± 6.91

Splenocyte #: n=7 for both male and 12 for both female groups

Immune subsets: n=7 for male vehicle, 6 for male fenofibrate, 9 for female vehicle and 7 for female fenofibrate

* indicates $p < 0.05$,** indicates $p < 0.01$ ^a indicates $p = 0.057$ by t-test.