

The Role of TGF- β and Angiotensin Receptor Blockers in Modulation of
Microglial Activation

By

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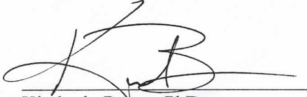
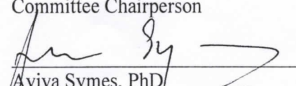
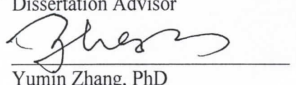
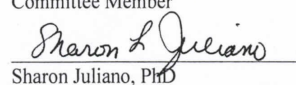
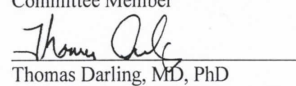
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DEDICATION

To my wife, Dr. Mrs. Yvonne Affram and my mother, Dr. Mrs. Kafui Affram

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ABSTRACT

The Role of TGF- β and Angiotensin Receptor Blockers in Modulation of Microglial Activation

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Chronic neuroinflammation is strongly associated with the development of neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Microglia, as the resident macrophages of the brain, are major regulators of the brain's inflammatory response and are chronically activated in many neurodegenerative diseases. Chronic microglial activation occurs despite the activity of several counter regulatory anti-inflammatory signals. Regulation of chronic microglial activation through augmentation of anti-inflammatory pathways should therefore be beneficial in ameliorating neurodegeneration. However, there is some evidence that anti-inflammatory pathways are inhibited in activated microglia. In this thesis, inhibition of the anti-inflammatory TGF- β signaling pathway as a potential cause of chronic microglial activation was examined. Using primary rat microglial cells we showed that the immune stimulator lipopolysaccharide (LPS) or the Alzheimer's related peptide, A β , reduced mRNA and protein expression of the TGF- β receptor, T β R1 and inhibited TGF- β stimulated gene expression. TGF- β mediated cell death of primary microglia was also inhibited by A β treatment. We showed that inhibition of the NF- κ B pathway, but not the MAP kinase or

IRF3 pathways resulted in a reduced ability of A β and LPS to restrict TGF- β signaling. Therefore, A β mediated inhibition of TGF- β signaling could contribute to chronic microglial activation. Development of strategies that can augment the anti-inflammatory effects of TGF- β signaling may be beneficial to treat neurodegenerative diseases.

Anti-inflammatory drugs may be useful to reduce chronic microglial activation and hence be potential therapeutics for neurodegenerative diseases. Retrospective studies examining patients taking the anti-hypertensive angiotensin receptor blocker drugs (ARBs), have shown reduced incidence of Alzheimer's disease compared to people taking other classes of anti-hypertensives. As ARBs have known to be anti-inflammatory in addition to their effects on blood pressure regulation we compared the anti-inflammatory effects of different ARBs on LPS-activated BV2 microglial cells. We found that several ARBs reduce LPS-mediated stimulation of BV2 cells, with telmisartan having the strongest anti-inflammatory activity. Telmisartan was also the only ARB to phosphorylate AMP Kinase and reduce mTOR phosphorylation. Inhibition of AMPK with compound C partially inhibited telmisartan's ability to reduce LPS mediated microglial activation. Additionally, telmisartan uniquely reduced microglial viability thus suggesting that telmisartan may reduce the microglial population to ameliorate chronic neuroinflammation. These data show that telmisartan has unique anti-inflammatory activity in microglia, and may therefore be a potential therapeutic to reduce chronic microglial activation and hence ameliorate detrimental pathology occurring in brains of patients with neurodegenerative diseases.

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CHAPTER 1: Introduction

ABBREVIATIONS:

Angiotensin receptor blockers (ARBs), Alzheimer's disease (AD), Central Nervous System (CNS), Transforming growth factor β (TGF- β), Lipopolysaccharide (LPS), Reactive oxygen species (ROS), Nitric Oxide (NO), Interleukin 1 β (IL1 β), Tumor necrosis factor α (TNF α), Interferon γ (IFN γ), Peroxisome proliferator-activated receptor γ (PPAR γ), Pathogen associated molecular patterns (PAMPs), Damage associated molecular patterns (DAMPs), Renin angiotensin system (RAS), Pattern recognition receptors (PRRs), Blood brain barrier (BBB), Angiotensin II (Ang II), Angiotensin type 1 receptors (AT1R), Angiotensin type II receptors (AT2R), Food and drug administration (FDA), Amyloid β (A β), Inducible nitric oxide synthase (iNOS), Toll-like receptors (TLRs), Interferon regulation transcription factor (IRF), Nuclear factor κ B (NF- κ B), calcium/ calmodulin dependent protein kinase kinase β (CaMKK β), heat killed listeria monocytogenes (HKLM), Tetramethylrhodamine, methyl ester, perchlorate (TMRE).

NEURODEGENERATIVE DISORDERS: A BRIEF OVERVIEW

Neurodegenerative diseases are a group of neurological disorders characterized by relentless irreversible decline in neurocognitive function and dementia as a result of chronic neuronal loss (215). The most prevalent neurological disorders include

Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (215). Alzheimer's disease (AD) however by far constitutes the majority of cases of neurodegenerative disorders (184; 218). The global population of people suffering from neurodegenerative disorders has been increasing (117; 222) due to increasing aging populations worldwide (218). According to the Alzheimer's Association, an estimated 5.4 million people live with AD in the United States and this number is projected to increase to 13 million by the middle of the century. Alzheimer's disease is one of the fastest growing causes of death and had a healthcare cost estimated at over 220 billion dollars in 2015 (8). The alarming epidemiology of neurodegeneration has made it a public health imperative to develop strategies that will reduce the burden of disease on the population.

Although the etiology of AD and other neurodegenerative disorders are usually idiopathic, there are several disease states that are known to predispose to neurodegenerative diseases. Many of these diseases are associated with chronic neuroinflammation (184; 219). Diabetes mellitus (65; 311), traumatic brain injury (185), brain infections (217; 221; 328) and vascular disorders (13) all increase the probability of development of neurodegeneration. Because AD and neurodegenerative disorders develop in older people, it is apparent that progressive and cumulative CNS insults play a role in their development. Predisposing conditions clearly impact the brain many years prior to development of symptoms (14; 169).

Chronic neuroinflammation therefore appears to be an important link between metabolic, traumatic, infectious or ischemic CNS insults and the subsequent development of neurodegeneration (48). The time between an injurious CNS event and the development of neurodegeneration thus provides a window of opportunity for the prevention or

amelioration of the development of neurodegenerative disorders by targeting chronic neuroinflammation.

NEUROINFLAMMATION

The CNS has been designated an immune “privileged site” due to limitation of access of circulating immune cells (10; 172). The blood brain barrier (BBB) formed by endothelial cells and supported by astrocytes helps to limit access to circulating inflammatory cells (172). Astrocytes also secrete anti-inflammatory mediators to reduce activation of immune cells like T-lymphocytes and macrophages that access the CNS (10; 210; 304). The presence of proinflammatory mediators like IL1 β , TNF α reactive oxygen and nitrogen species due to CNS inflammation all cause neurotoxicity (57; 128). The main cells involved in neuroinflammation are the microglia, (137) which work in association with astrocytes and neurons (55; 129). Circulating cells like neutrophils, monocytes and lymphocytes also participate in neuroinflammation after CNS injury (10; 304).

MICROGLIA

Microglia are the principal immune cells of the central nervous system (137). The origin of microglia has been a topic for debate however, current consensus accepts that they are mainly derived from the yolk sac during embryonic development and migrate to populate the developing CNS, although contribution from circulating monocytes may occur under some circumstances (7; 95). Microglia constitute 5-20% of the CNS cell population (137) and remain resident in the CNS for life with negligible turnover with circulating monocytes (95; 137). Microglia being long-lived cells in the brain has potential pathological implications as deleterious changes may persist for the life of an individual and contribute to neurodegeneration.

Microglia are the smallest of all CNS cells but exhibit extensive morphological and functional plasticity under different conditions (29; 137; 305). In the unperturbed brain,

microglia are small with ramified processes, in constant surveillance of the CNS (205). However when pathological changes occur, the microglia become activated, their morphological and functional states are altered to mount an inflammatory response to protect the CNS (122). The activation states of microglia have been simplistically designated M1 and M2 (220).

The M1 or classically activated state of microglial activation is associated with phenotypic transformation of microglia to more amoeboid shapes with increased expression of MHC II and proinflammatory cytokines like interferon gamma ($\text{IFN}\gamma$), interleukin 1 beta ($\text{IL1}\beta$), tumor necrosis factor alpha ($\text{TNF}\alpha$) and inducible nitric oxide synthase (iNOS) along with increased reactive oxygen species (ROS) release (29; 122). Although the M1 state is targeted at protecting the CNS during pathological alterations, the release of proinflammatory cytokines and ROS has detrimental effects to neurons (29; 139). Thus microglia remaining chronically in M1 activation states can predispose to neurodegeneration. The M2 or alternative activation state is associated with CNS repair and cleaning up of debris after CNS pathology (122; 139). The M2 state is typically induced by anti-inflammatory cytokines like IL4, IL10 and $\text{TGF-}\beta$ (51). The anti-inflammatory mediators cause microglia to secrete less proinflammatory mediators and ROS and rather increase their anti-inflammatory cytokine release along with increased phagocytosis. The M2 phenotype is thus thought to be beneficial to CNS health (122; 139).

The M1/M2 designation of activated microglia is however not that clear cut in reality as it is described. Currently, it is thought that activated microglia exhibit phenotypes that fall along a spectrum of which the M1 and M2 designation represent polar extremes (139; 198). The ascribed beneficial effect of M2 in contrast to M1 also does not universally

hold as it has been suggested that M1 phenotypes are more beneficial in CNS tumors in contrast to M2 states (284). The detrimental or beneficial effects of microglial activation states may therefore be context dependent.

Besides being involved in inflammation, microglia have also been shown to participate in pruning of neuronal synapses and help to facilitate synaptic transmission (305). They also facilitate neuronal proliferation and differentiation in some parts of the brain (95; 124).

MICROGLIAL ACTIVATION AND CHRONIC INFLAMMATION IN NEURODEGENERATIVE DISORDERS

Nearly all neurodegenerative diseases are associated with chronic neuroinflammation (97). A hallmark of most neurodegenerative disorders is the accumulation of abnormal proteins within neurons and the CNS parenchyma (97; 103). In Alzheimer's disease, the abnormal proteins include amyloid- β (A β) peptides which agglomerate to form oligomers, fibrils and plaques (97; 103). In Parkinson's disease α -synuclein proteins accumulate (309). This abnormal protein accumulation indicates a difficulty in pathological protein clearance in these disorders (67). Abnormally accumulated proteins like α -synuclein, tau and A β directly activate microglia which release proinflammatory mediators deleterious to neurons (97). It is therefore clear that chronic microglial activation and the ensuing neuroinflammatory process play a significant role in the disease pathogenesis in neurodegeneration. Many therapeutic strategies for neurodegenerative disorders are thus aimed at reducing abnormal protein accumulation and amelioration of chronic microglial activation and neuroinflammation (31).

MOLECULAR PATHOGENESIS OF NEUROINFLAMMATION.

Although microglia are central to neuroinflammation, nearly all CNS cells participate in neuroinflammation. Injury to CNS tissue whether via trauma, ischemia, infection or metabolic alterations results in the exposure to dead and dying CNS cells and their components which are detected by innate immune system receptors, collectively called pattern recognition receptors (PRRs), in microglia (135). Parts of damaged tissue recognized by microglia are referred to as damage associated molecular patterns (DAMPs) (156). Examples of DAMPs include A β peptides, α -synuclein, advanced glycation end products, HMGB1, ATP and damaged mitochondria (11; 263; 291). Infectious agents and their components are referred to as pathogen associated molecular patterns (PAMPs) (230). Examples of PAMPs include lipopolysaccharide (LPS), lipoteichoic acid and live bacteria (230).

The major PRRs involved in microglial activation include toll-like receptors (TLRs), RIG-1 like receptors, Nod-like receptors and AIM2-like receptor (135; 230) and scavenger receptors like receptors of advanced glycation end products (RAGE), and CD36 (138; 292). Of these PRRs, Toll-like receptors are the best characterized (135). Signaling through TLRs and other PRRs results in downstream effects that ultimately lead to the secretion of proinflammatory mediators like nitric oxide (NO), ROS, IL1 β , IFN γ , and TNF α (46; 230).

While neuroinflammation consistently occurs with CNS injury during trauma, infection and ischemia, some subtle physiological changes can also lead to neuroinflammation. Metabolic alterations (329), increased blood pressure (70; 235) and even abnormally increased neuronal firing (304) could predispose to injury in all body systems including the CNS. It is well established that metabolic diseases like diabetes

mellitus is associated with systemic as well as neuroinflammation (251). While increased blood pressure by itself can cause injury to vessels and tissues, the signaling pathways involved in maintaining high blood pressure also activate proinflammatory pathways (19).

TOLL LIKE RECEPTOR SIGNALING IN MICROGLIA

There are 10 identified TLRs in humans and 12 identified in mice (135). TLRs are subdivided into cell surface TLRs and intracellular TLRs. TLR4 is a cell surface TLR which recognizes bacteria LPS (38) and the DAMP A β . TLR2 is also a cell surface TLRs which recognizes lipoteichoic acids, zymosan and listeria monocytogenes (135; 268).

On binding to the appropriate ligand, TLRs activate downstream signaling by recruiting adaptor proteins to their intracellular domains, which determine the ultimate cellular effect. MyD88 is an adaptor protein which when recruited results in activation of the MAPK or NF- κ B pathway (135) (Figure 1). NF- κ B is mainly locked in the cytoplasm by I κ B in the absence of inflammatory stimuli. On activation of the MyD88 pathway, I κ B is phosphorylated by I κ B kinase and targeted for degradation, hence releasing NF- κ B to translocate into the nucleus to effect regulation of inflammatory gene transcription. The alternate MyD88 independent pathway utilizes the adaptor proteins Tram and TRIF which leads to the activation of TBK1 and downstream Interferon regulation transcription factor (IRF3) translocating into the nucleus to induce type 1 interferon genes (5; 135) (Figure 1).

THE RENIN ANGIOTENSIN SYSTEM

One of the endogenous systems which when dysregulated can predispose to systemic and neuroinflammation is the renin angiotensin system (RAS) (252). The RAS functions primarily regulate blood pressure and maintain cardiovascular homeostasis (250; 293). The RAS is believed to be an evolutionarily conserved repair mechanism as, in

association with its blood pressure maintaining effects, it is also thrombogenic, proliferative as well as fibrogenic all of which are processes potentially involved in tissue repair following injury and fluid conservation (293). It is thought that the RAS system may thus have conferred some advantage in the evolutionary past, some of which may be detrimental in the present (92; 283).

The RAS consists of a series of enzymatic cleavage of angiotensinogen to angiotensin II (Ang II), the main effector peptide of the system (Figure 2). Angiotensinogen, which is mainly produced in the liver, is cleaved to angiotensin I by renin followed by conversion of Angiotensin I to Ang II by angiotensin converting enzyme (ACE) (250). Other cleavage products of angiotensinogen, angiotensin I and Ang II including Angiotensin 1-7 (130) Angiotensin 1-9 (207), and Angiotensin III (142) have been described but their effects are relatively less well characterized. Thus far, Ang II is known to mediate most of the vascular regulatory effects of the RAS. Ang II can induce the release of aldosterone from the adrenal medulla, reduce fluid loss in the kidney and cause vasoconstriction to elevate blood pressure (250).

Ang II serves as ligand to two receptors, Angiotensin type 1 receptors (AT1R) and angiotensin type 2 receptors (AT2R) (6; 69). AT1Rs can be further subdivided into AT1Ra and AT1Rb in rodents but not in humans (69). Generally, AT1Rs mediate most of the observed effects of Ang II including the vasopressor effects, aldosterone release, proinflammatory and fibrogenic effects (19; 69). Activation of AT1Rs can activate proinflammatory pathways like the NF- κ B and MAPK pathways and also induce the generation of ROS (19; 80). On the other hand, AT2Rs usually tend to have the opposite

effects of AT1Rs. AT2Rs mediate anti-inflammatory, apoptosis and antiproliferative effects (69; 106).

RAS AND NEUROINFLAMMATION

Although the RAS was initially described in non-CNS tissues, subsequent research demonstrated a complete RAS in the CNS (154; 186). The brain specific RAS is involved in central regulation of blood pressure (186). Various brain cells express different components of the RAS. Astrocytes have the highest expression of angiotensinogen while neurons express AT1Rs (186). The expression of renin was found to be low in CNS and it is believed that cathepsinD may complement the function of renin in cleavage of angiotensinogen (186). ACE is abundant in the CNS and is expressed in neurons and microglia (23; 143; 186). Activation of the RAS in the brain, like in non-CNS tissues, can activate proinflammatory pathways and induce ROS release (42; 252). Not surprisingly, increased AT1R activation worsens neuroinflammation and neurodegeneration (19). Activation of AT1Rs can lead to disruption of the BBB and thus also induce brain inflammation (25; 228).

The RAS however appears to have a protective arm in activation of AT2Rs. Activation of AT2Rs has been associated with anti-inflammatory and anti-proliferative effects that are potentially neuroprotective (104; 186). It has also been shown that Ang 1V, a cleavage product of Ang II, improves memory and cognitive function (186; 296). The RAS therefore has beneficial and detrimental effects on the CNS that need to be recognized. The prevailing consensus however is that over activation of the RAS is generally detrimental (90) and therefore current efforts are geared towards reducing activation of the RAS system in the brain (228; 273).

PATHWAYS INVOLVED IN INHIBITION OF MICROGLIAL ACTIVATION

Several signaling pathways are present in immune cells like microglia that serve to inhibit inflammatory responses in order to reduce the deleterious effect of inflammation on tissues. As part of the inflammatory response some of the cytokines released have anti-inflammatory effects. Examples of anti-inflammatory cytokines include TGF- β (26), IL10 (56), IL4 (174) and IL13 (178). There are also intracellular protein regulators that can be activated by several different endogenous and exogenous factors that activate anti-inflammatory cellular processes. These pathways include the PPAR γ (132) pathway and the AMPK pathway (232).

TGF- β SIGNALING

TGF- β is a pleiotropic cytokine with diverse effects depending on tissue type and context (177). TGF- β has anti-inflammatory, anti-proliferative, proapoptotic and profibrotic effects in many tissues (177; 182). TGF- β signaling is initiated by the binding of TGF- β to the serine threonine receptor T β R2 which then phosphorylates and activates T β R1 in a heteromeric T β R2/ T β R1 complex (295) (Figure 3). In the canonical signaling pathway, T β R1 activates receptor associated Smad proteins (R-Smad) 2 and 3 which associate with co-smad Smad4, thus facilitating their nuclear localization and regulation of gene transcription (182). TGF- β signaling through pathways distinct from Smad activation have also been characterized and are referred to as non-canonical pathways. These include activation of MAPK pathway, RHO-like GTPase pathway and the PI3K pathways (325).

TGF- β signaling pathways are negatively regulated via several mechanisms. Induction of inhibitory Smad proteins Smad6 and 7 constitute a negative feedback loop for the regulation of TGF- β signaling. Smad7 inhibits downstream activation of r-Smads by

T β R1 (315). Smad6 inhibits TGF- β signaling but is more commonly associated with inhibition of the related BMP pathway (116). SnoN and Ski are two other proteins that negatively regulate TGF- β signaling by modulating Smad mediated actions (182). Also the decoy receptor Bambi which can bind TGF- β but cannot activate T β R1 to effect signaling is also involved in negative regulation of T β R1 signaling (84; 182). E3 ubiquitin ligases are another class of proteins that are involved in regulation of TGF- β signaling. Smurf 1 and Smurf2, both E3 ligases, mediate the degradation of Smad proteins and TGF- β receptors to negatively regulate the signaling pathway (126). Canonical TGF- β signaling is positively regulated by increase in expression of TGF- β receptors by TGF- β (194). Also Arkadia, an E3 ligase, can ubiquitinate and degrade Smad7, Ski and SnoN to prevent inhibition of TGF- β signaling (126).

Given that TGF- β plays an inhibitory role in inflammation, inhibition of TGF- β signaling can lead to excessive inflammatory states. Dysregulation of TGF- β signaling has been implicated in inflammatory disorders like inflammatory bowel disease (89). Indeed TGF- β KO mice suffer early death due to severe systemic inflammation (148). Recent studies have also shown that TGF- β signaling is dysregulated in the CNS in neurodegenerative disease (62; 276; 300). Tesseur *et al* (2006) showed that TGF- β signaling in human neurons is impaired as a result of reduced expression of T β R2 in neurons in Alzheimer's disease (262). It has also recently been shown that impairment of TGF- β signaling results in striatal neuronal degeneration in an animal model of Parkinson's disease (261). Strategies aimed at augmenting CNS TGF- β signaling in chronic inflammatory states may thus be beneficial in neurodegenerative disorders.

CROSSTALK BETWEEN PROINFLAMMATORY PATHWAYS AND TGF- β SIGNALING

The TGF- β signaling pathway cross talks with several other signaling pathways to modify cellular responses (108). By interacting with other pathways, TGF- β can either modify them or be modified. TGF- β mediates its anti-inflammatory effects in part by antagonizing proinflammatory signaling pathways. TGF- β has been shown to inhibit TLR4 signaling through Smad6 induction. TGF- β induced Smad6 mediates the degradation of MyD88 by recruiting an E3 ligase (160). Smad6 also interacts with the adaptor protein pellino-1 to inhibit interleukin 1 receptor-associated kinase 1 in HEK293 cells (IRAK1). IRAK1 is involved in IL1 β mediated induction of inflammation (54). TLR4 signaling has also been shown to inhibit TGF- β signaling. In RAW264.7 macrophages, Kim *et al* showed that TGF- β mediated gene induction can be inhibited by LPS via the ERK pathway (141). Also NF- κ B mediated inhibition of TGF- β signaling has been demonstrated via induction of smad7 expression (27).

Interaction between TLR4 and TGF- β signaling is not always antagonistic. TLR4 activation caused down regulation of the TGF- β pseudo receptor and antagonist Bambi leading to up regulation of TGF- β mediated fibrosis in the liver (241). TGF- β can also mediate inflammation under certain circumstances. TGF- β was shown to worsen inflammation in cutaneous wound healing in mice (280) and increase ROS release in liver (316). The proinflammatory effect of TGF- β is cell specific and context dependent. It has been shown that TGF- β in the presence of IL6 can promote the differentiation of T helper 17 cells which can increase inflammatory responses (234). In CD8 T cells, the effect of TGF- β on inflammatory response is dependent on previous exposure of the cells to antigens. TGF- β increased IFN γ and IL17 release in memory CD8 T cells while reducing proinflammatory cytokine release from naive CD8 T cells (87).

Previous studies in our lab have also shown the LPS treatment of microglia inhibits TGF- β signaling (194) however the molecular mechanisms were not elucidated. Given that there is increased and persistent microglial activation in many neurodegenerative disorders (31), understanding the link between microglial activation and impairment of TGF- β signaling could reveal potential therapeutic targets.

PPAR γ PATHWAY

PPAR γ are nuclear receptors involved in regulation of lipid and glucose metabolism (3; 73; 179). PPAR γ receptors are abundant in adipocytes, muscle and macrophages (179). Activation of these receptors has been associated with down regulation of proinflammatory genes (179). The endogenous ligands for PPAR γ receptors are prostaglandin metabolites derived from fatty acids and eicosanoids (105; 179). Binding of ligands to PPAR γ leads to homodimerization or heterodimerization with other nuclear receptors like the retinoic acid receptor prior to regulation of gene transcription in the nucleus (298).

Thiozolidindiones or glitazones are a group of PPAR γ ligands that have been developed for the management of diabetes mellitus (21). These drugs have also been shown to have anti-inflammatory effects both dependent and independent of PPAR γ (199). Glitazones have been shown to reduce proinflammatory cytokine release from microglia and macrophages neuroinflammation and ameliorate the development of neurodegeneration (294).

AMPK PATHWAY

The AMPK pathway is a central regulator of metabolism and growth of cells (191). AMPK is activated in the presence of cellular stress and starvation to maintain energy metabolism (Figure 4). Activation of AMPK is increased when AMP/ATP ratio in cells is

increased (191). The AMPK pathway is a therapeutic target for diabetes mellitus where the biguanide metformin, an AMPK activator, is used as a first line drug (120). Activation of the AMPK pathway is also associated with inhibition of inflammation and neuroprotection. Activators of AMPK have been shown to reduce proinflammatory mediators in activated microglia (44). The anti-inflammatory effects of AMPK appear to be mediated by inhibition of mTOR, a potent inhibitor of autophagy, thus resulting in autophagy induction (260; 308). Several recent studies have shown that induction of autophagy results in inhibition of inflammatory processes (35; 114). The AMPK/mTOR/Autophagy pathway may therefore be an important therapeutic target for chronic neuroinflammation and neurodegenerative disorders.

AUTOPHAGY, APOPTOSIS AND CELL DEATH IN THE REGULATION OF NEUROINFLAMMATION

Autophagy is a cellular process involved in the degradation of cellular content for the maintenance of cellular homeostasis during nutrition stress (68; 98). Autophagy is also employed in eliminating abnormal proteins and organelles that would otherwise cause cell damage (98; 206). The process of breaking down cellular components to simple molecules helps to maintain substrates for metabolism (149; 227). However, if autophagy becomes excessive, the process can lead to cell death (98; 269).

The autophagic process involves the formation of phagophores and autophagosomes, which enclose the cargo to be broken down (Figure 5). Membrane bound autophagosomes fuse with lysosomes to form autolysosomes in which the contents are degraded by proteolysis (98). Many proteins are involved in the autophagic process. Beclin1 is involved in initiating phagophore formation in association with several autophagy (ATG) proteins (98). LC3B, a ubiquitous cytoplasmic protein is incorporated

into the phagophore membrane when autophagy is induced. LC3B prior to autophagy induction is not lipid conjugated and referred to as LC3B1. Before LC3B is incorporated into the phagophore membrane, it is conjugated with phosphatidylethanolamine and converted to LC3BII (17; 98). The difference in molecular weight between LC3BI and II has proved a useful marker for assessment of autophagy induction in cells (17).

Autophagy is associated with anti-inflammatory effects in part by targeting of inflammasomes which are responsible for generation of IL1 β by immune cells (59). Induction of autophagy results in targeting of inflammasomes for degradation via ubiquitination (53; 327). Also autophagy can augment microglial clearance of A β deposits and this can also reduce the amount of A β plaques that can incite neuroinflammation (53). Given that the autophagic process involves degradation of cellular components, accumulated intracellular toxic proteins can be degraded as well and thus autophagy provides a potential beneficial effect to neurons which could get damaged by toxic intracellular accumulations like abnormal tau and A β (158).

A mechanism by which immune activation can be modulated is by induction of immune cell death (326). Many anti-inflammatory cytokines like IL4 (176), IL13 (317) and TGF- β (240) can modulate inflammation by inducing death in immune cells. The increased number of activated microglia in neurodegeneration may be an indication of impairment of death of these cells and strategies that can help reduce the population of activated microglia could be beneficial.

Recent experiments have sought to employ strategies that reduce the population of microglia in the murine brains in order for possible application in neuropathology. Elmore *et al* developed a murine model in which microglia were depopulated in the brain with

colony stimulating factor 1 inhibitor and observed no significant detrimental effect on function. Although 99 percent of the microglia were eliminated, a microglial stem cell niche repopulated the CNS within a week (74). It would be interesting to see how eliminating chronically activated microglia temporarily will be beneficial in AD. Induction of immune cell death is however a therapeutic strategy in a chronic inflammatory disorders like inflammatory bowel disease using glucocorticoids (33).

THERAPEUTIC STRATEGIES FOR NEURODEGENERATION TARGETING NEUROINFLAMMATION

After initial injury to the CNS, the inevitable death of local cells cannot be avoided. However, in many cases, long term secondary inflammation which can linger on for years with ongoing chronic neuronal loss can occur (83). This second phase of low intensity chronic neuroinflammation is believed to be the link between an injurious CNS event and neurodegeneration later in life (83). Although classic neurodegenerative disorders may not necessarily have identifiable initiating events, their pathogenesis is however linked to chronic neuroinflammation (303). The period of chronic neuroinflammation preceding development of neurodegeneration provides a window of therapeutic opportunity. It has been proposed that interventions aimed at ameliorating chronic neuroinflammation may be beneficial in reducing neurodegeneration (57).

Given that chronic microglial activation is central to chronic neuroinflammation, strategies to ameliorating microglial activation are important therapeutic aims. Currently, it is not completely understood how microglia can remain activated for long periods after a CNS injury. Some studies have shown inhibition of anti-inflammatory pathways in microglia after activation. One study showed that the CD200 receptor, which has its ligand CD200 on neurons and is involved in keeping microglia quiescent, is down regulated after

induction of neuroinflammation, thus keeping microglia chronically activated (181). We have also previously shown that TGF β signaling is reduced after LPS treatment of primary microglia (194). Inhibition of microglial TGF β signaling has implications for chronic microglial activation, as it is a key anti-inflammatory pathway which when compromised can leave proinflammatory pathways unopposed. Identification and interruption of signaling pathways that could inhibit anti-inflammatory pathways could be important therapeutic strategies for neurodegeneration.

Another strategy for the development of interventions for neurodegeneration is targeting of chronic neuroinflammation by repurposing of prior FDA approved drugs with known anti-inflammatory and potential neuroprotective effects (4). Several trials with PPAR γ agonists, dapsone NSAIDs, hydroxychloroquine and glucocorticoids have however not been successful in reducing the occurrence of neurodegeneration (4; 278). Nonetheless many candidate drugs are still in various stages of preclinical and clinical trials aimed at ameliorating neurodegeneration (277; 278; 289).

Recently, interest has been growing in the potential therapeutic effects of regulating the RAS in the brain (228). We have previously shown that the angiotensin receptor blockers (ARBs) candesartan and telmisartan have neuroprotective effects in a mouse model of traumatic brain injury (272; 273). A further elucidation of the molecular mechanisms of ARBs in reducing microglial activation and neuroinflammation will be important for a better understanding of the therapeutic potential of ARBs and possible detrimental effects.

ANGIOTENSIN RECEPTOR BLOCKERS

Angiotensin receptor blockers are FDA approved drugs primary used in treatment of hypertension and other cardiovascular disorders (134). Currently there are 8 FDA approved ARBs in clinical use. They are candesartan, losartan, irbsartan, valsartan, eprosartan, olmesartan, telmisartan and azilsartan (134). Besides their effects on blood pressure and renal water regulation, many ARBs have been shown to have anti-inflammatory effects and thus led to the idea that they could be of benefit in chronic neuroinflammation and neurodegenerative disorders (228). Epidemiological evidence suggests that patients prescribed ARBs for hypertension have reduced incidence of neurodegenerative disorders (63). Because all the ARBs block AT1R signaling (134), the initial assumption should be that the anti-inflammatory effects of the ARBs are AT1R dependent. However, it has been shown that many of the ARBs activate anti-inflammatory pathways independent of AT1R blockade. These pathways include the PPAR γ and AMPK pathways. Of the ARBs, losartan, telmisartan, candesartan and irbsartan have partial PPAR γ activity (76). Telmisartan however has the strongest PPAR γ activation ability. Telmisartan has also been shown to activate the AMPK pathway (93).

ARBs AS THERAPY FOR NEUROINFLAMMATION AND NEURODEGENERATION

The ARBs have been shown to be neuroprotective in several studies (228; 273). Since ARBs are primarily used in treatment of hypertension and other cardiovascular diseases they can all indirectly protect the brain by ameliorating the deleterious effects of cardiovascular diseases on CNS cells (71; 196). However, direct activation of AT1R in the brain can also have deleterious effects independent of the cardiovascular system (125; 196). The ARBs with partial PPAR γ activity potentially have additional neuroprotective

mechanisms, as chronic neuroinflammation and neurodegeneration are associated with dysregulation in metabolism (65; 155).

A limitation of the ARBs in having direct neuroprotective effects is their limited ability to cross the blood brain barrier in the absence of BBB breakdown like in trauma, ischemia or inflammation (134). Telmisartan however happens to be the most BBB permeable of all the ARBs and has been administered intranasally to reduce AD pathology in a murine model (134; 266). In a study using rats, telmisartan was detected in the cerebrospinal fluid 8 days after oral administration (99). Telmisartan's increased ability to cross the BBB over the other ARBs is in consonance with it being more lipophilic (190).

Of the ARBs, candesartan and telmisartan have been most studied in terms of their neuroprotective effects. Candesartan was shown to reduce LPS induced neuroinflammation in mice (18). Also candesartan and telmisartan were efficacious in reducing neuroinflammation and behavioral deficits after traumatic brain injury in mice (272; 274). Candesartan also reduced microglial activation mediated by α -synuclein in a model of Parkinson's disease (61). Recently telmisartan was shown to have therapeutic effect in animal models of Alzheimer's disease and other models of neuroinflammation (152; 266; 267). Currently, both candesartan (112) and telmisartan (289) are in clinical trial for prevention of Alzheimer's disease in humans. The telmisartan trial is being targeted at mainly African-American populations while candesartan is in clinical trial to assess its ability to reduce mild cognitive impairment in aging (289).

Of the ARBs, only telmisartan has so far been shown to activate the AMPK pathway (312) and induce autophagy (163; 246). Given that AMPK activation and induction of autophagy are usually anti-inflammatory and neuroprotective (191),

telmisartan may have additional pathways for neuroprotection that may be lacking in other ARBs. Lastly, telmisartan treatment is associated with induction of apoptosis in several cells including immune cells (147). Induction of apoptosis in microglia by telmisartan may present another potential therapeutic mechanism in neuroinflammation.

CONCLUSION

Chronic neuroinflammation plays a central role in the development of neurodegenerative diseases and therefore finding strategies that can ameliorate long lasting CNS inflammation is a critical therapeutic goal. In this thesis I assessed two different strategies aimed at understanding mechanisms of chronic neuroinflammation and to ameliorate neuroinflammation in order to reduce or prevent the development of neurodegeneration.

In chapter 2, I sought to answer the question why microglia have the tendency to remain activated for long periods of time following an initial CNS insult and hence predispose to neurodegeneration. Having previously shown that the key anti-inflammatory TGF- β signaling pathway is inhibited in microglia with LPS treatment, I sought in the study to assess whether TGF- β signaling will be down regulated in A β and HKLM treated microglia, modeling for Alzheimer's disease and bacterial meningitis respectively. My hypothesis was that A β in Alzheimer's disease and HKLM would both down regulate TGF- β signaling in microglia as was observed with LPS treatment. I further assessed some molecular mechanisms by which TGF- β signaling is antagonized by the proinflammatory mediators.

The third chapter was focused on comparing the anti-inflammatory properties of different ARBs in microglia and further assessing the properties of telmisartan as a unique

ARB with both anti-inflammatory, autophagy inducing and proapoptotic affects. Here, I hypothesized that telmisartan has more potent anti-inflammatory effects compared to the other ARBs and involves telmisartan's ability to activate AMPK and induce autophagy.

Overall, we demonstrated that regulation of microglia for therapeutic ends can be approached by studying mechanisms by which microglia can become chronically activated by inhibition of anti-inflammatory pathways like TGF- β . We also show that telmisartan has unique cellular effects among the ARBs which include activation of AMPK, autophagy and cell death in microglia, mechanisms that explains its increased anti-inflammatory effects.

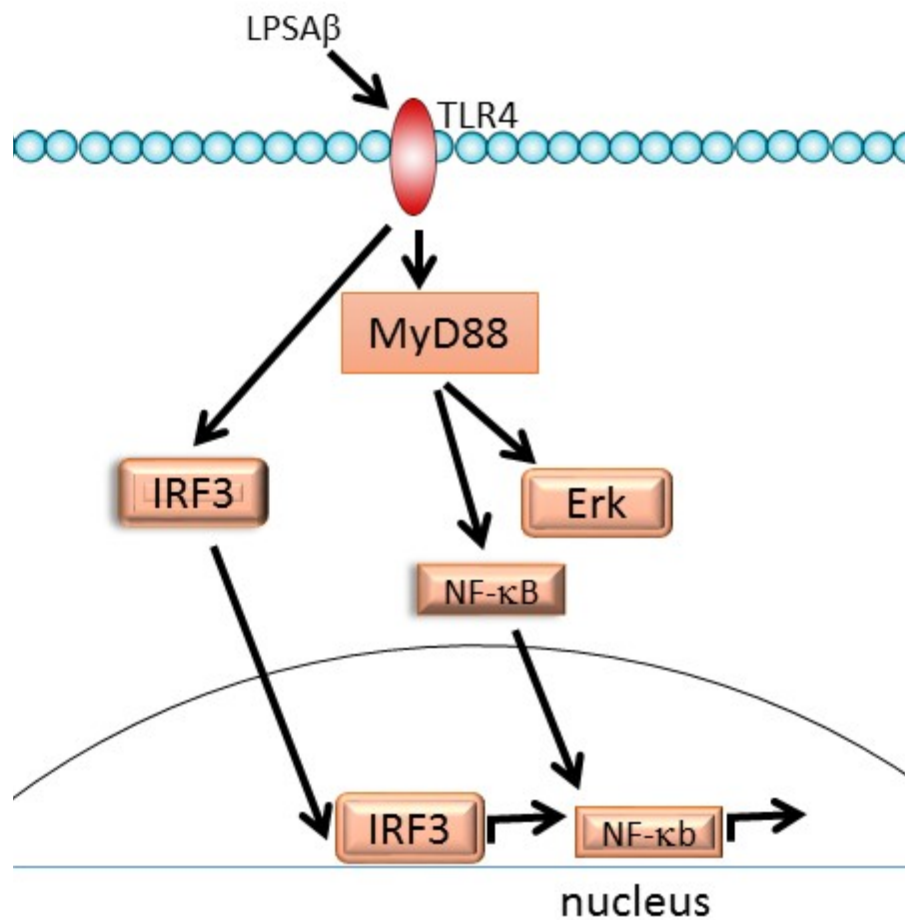


Figure 1. TLR4 signaling pathway

LPS or A β bind TLR4 and activate downstream pathways which are MyD88 dependent or MyD88 independent. MyD88 activates the Erk and NF- κ B pathways. Activation of IRF3 is independent of MyD88 and results in induction of type one interferon genes.

Renin Angiotensin System

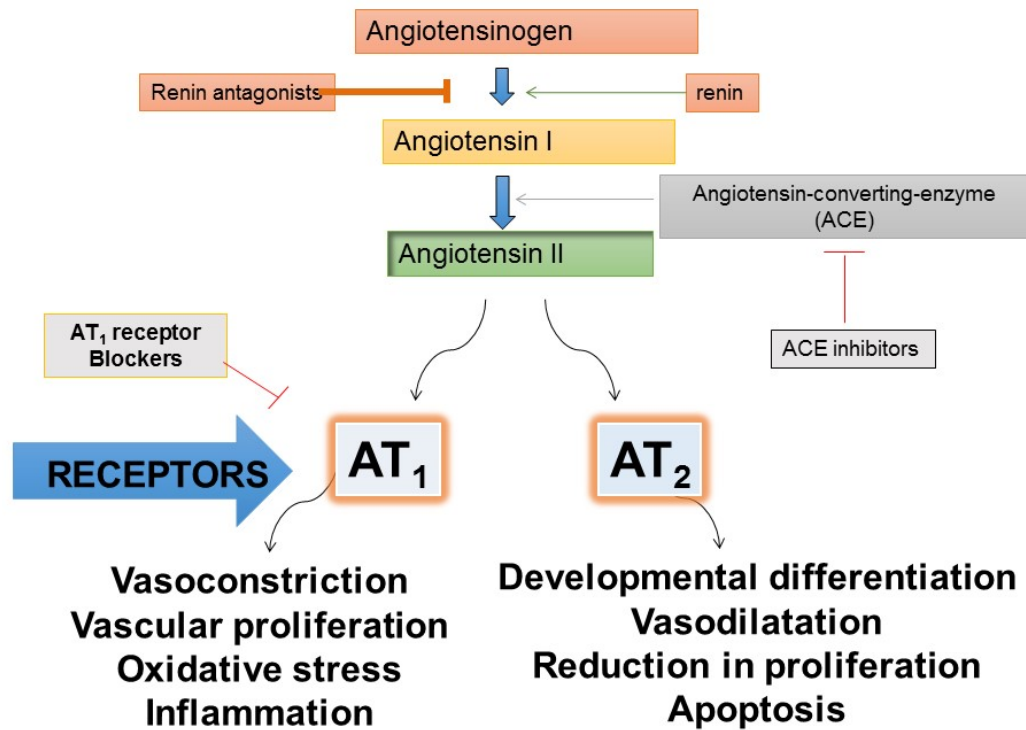


Figure 2. Renin Angiotensin System

The renin angiotensin system is initiated by cleavage of angiotensinogen to angiotensin I by renin. Angiotensin I is further cleaved into the main effector peptide, angiotensin II, by angiotensin converting enzyme (ACE). Angiotensin II acts on 2 main receptors, angiotensin type 1 receptor (AT1R) and type 2 receptor (AT2R.). AT1Rs mediate vasoconstriction and inflammation while AT2Rs mediated vasodilation and anti-inflammatory effects. AT1R is blocked by AT1 receptor blockers.

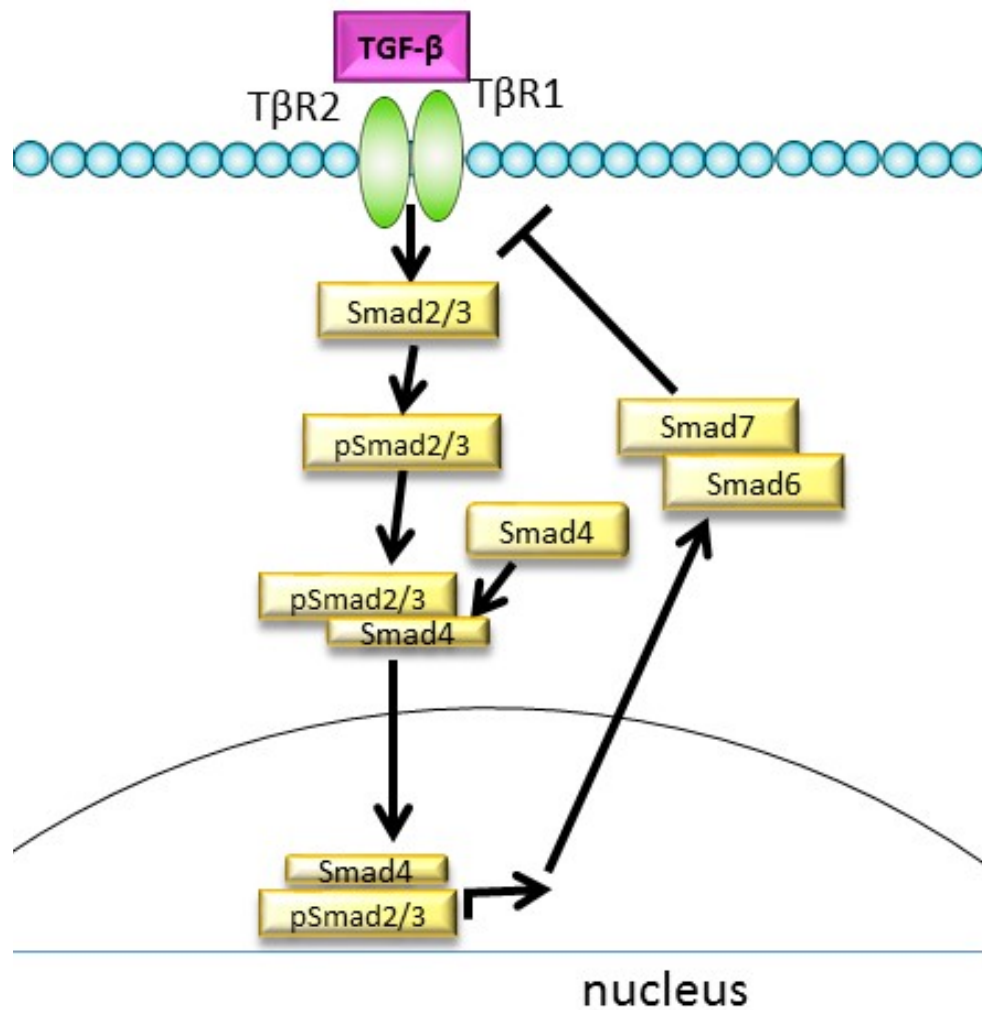


Figure 3. TGF-β signaling pathway

TGF-β binds to TβR2 which binds and activates with TβR. TβR phosphorylates r-Smads Smad2/3 by phosphorylation. R-Smads then combine with co-smad, Smad4 and translocates into the nucleus to regulate gene transcription. Induction of inhibitory Smads, Smad6 and 7 negatively regulates TGF-β signaling.

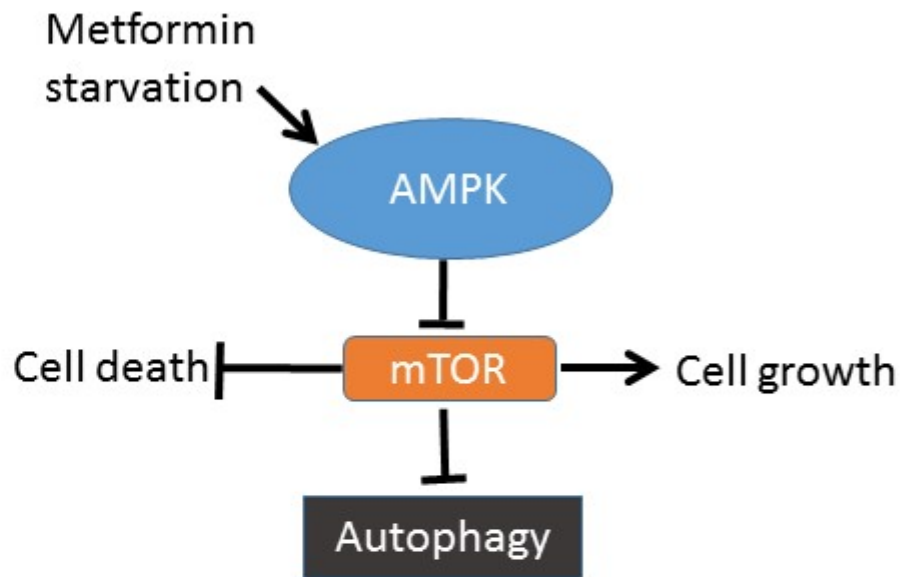
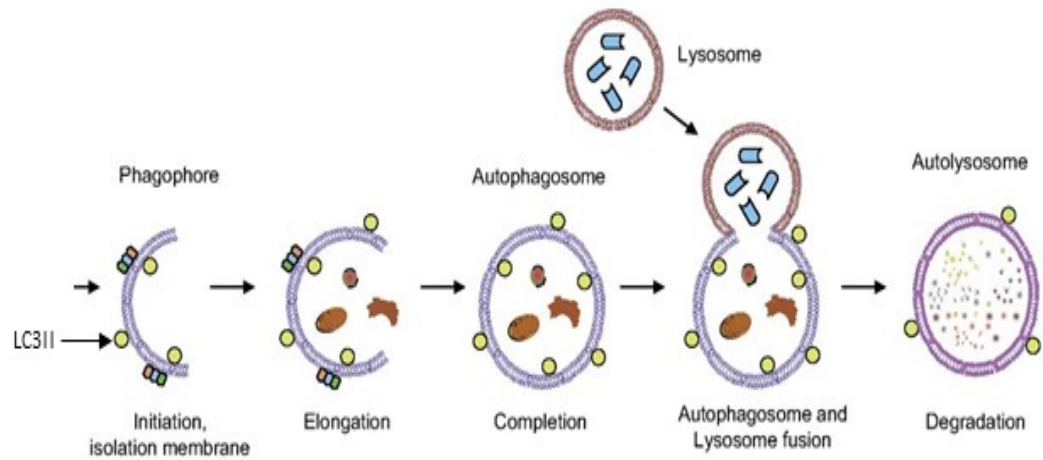


Figure 4. AMPK/mTOR/Autophagy pathway

AMPK is activated by several stimuli including nutrient deprivation and metformin. AMPK activation inhibits mTOR activation. mTOR induces cell growth and inhibits cell death and autophagy.



(Nakahira *et al* 2013)

Figure 5. Autophagic flux

Autophagy begins by the formation of a phagophore in association with ATG proteins including LC3II. Phagophores elongate and enclose the relevant cargo to form an autophagosome. Autophagosomes fuse with lysosomes to form autophagolysosomes followed by degradation.

CHAPTER 2: Microglial Activation Results in Inhibition of Canonical TGF- β Signaling

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ABSTRACT

Chronic inflammation mediated by persistent microglial activation is associated with the pathogenesis of neurodegenerative diseases. The mechanisms underlying chronic microglial activation are poorly understood. We have previously shown that anti-inflammatory TGF- β signaling is inhibited in LPS treated microglia. In this study we assessed the generic nature of inhibition of TGF- β signaling in activated microglia using different disease related microglial activators. We examined the effects of Amyloid β (A β) (1-42) or heat killed listeria monocytogenes (HKLM) on the TGF- β signaling pathway in primary rat microglia. We found that A β (1-42) oligomers and HKLM, in addition to LPS, suppressed TGF- β signaling in part through reducing expression of T β R1 mRNA encoding the TGF- β Receptor 1 in primary microglia. A β (1-42) and LPS also prevented induction of TGF- β induced genes in primary microglia. Additionally, A β (1-42) rescued primary microglia from TGF- β mediated cell death without increasing cell proliferation. Blockage of NF- κ B signaling, but not ERK or IRF3 pathways, inhibited A β (1-42) and LPS mediated reduction of T β R1 mRNA. Finally, LPS and A β (1-42) induced transient up regulation of mRNAs encoding SnoN and Bambi, inhibitors of TGF- β signaling. Our data indicate that one mechanism through which activators may prolong microglial stimulation is through direct inhibition of anti-inflammatory signaling. A more detailed understanding of the interaction between inflammatory and anti-inflammatory pathways may reveal potential

targets for ameliorating chronic inflammation and hence speed the development of therapeutics to address neurodegenerative diseases.

INTRODUCTION

Chronic neuroinflammation, whether induced by injury, metabolic derangements or deposition of abnormal proteins, is strongly associated with the development of neurodegenerative disorders like Alzheimer's and Parkinson's disease (10; 107; 270). The strong association between chronic neuroinflammation and neurodegeneration makes amelioration of neuroinflammation a therapeutic target for neurodegenerative disorders (49; 57). However, little is known about the molecular mechanisms that mediate prolonged neuroinflammation.

Microglial cells, the resident macrophages of the central nervous system (CNS), are key to the development and maintenance of neuroinflammation. Microglia have various morphological and functional forms influenced by the environment of the CNS. In an unperturbed CNS, resting microglia are small with ramified processes that constantly survey the CNS milieu (137; 173). Following pathological alterations, microglia become activated and range in form and function from cells that actively secrete proinflammatory mediators (M1 activation) to anti-inflammatory (M2) states with phagocytic and potentially neuroprotective effects (107; 137). Microglia tend to remain chronically in the M1 activated state following CNS injury, continually releasing proinflammatory and neurotoxic cytokines (217; 297). This chronic activation state perpetuates chronic neuroinflammation in the CNS that increases the risk of neurodegeneration (151; 171; 181).

Currently, we do not understand why microglia may remain chronically activated after a CNS injury. In non-CNS tissues like the liver and the gut, acute inflammatory processes typically resolve in response to regulation by anti-inflammatory mediators like TGF- β and IL10 (52; 223). However, although these anti-inflammatory mediators are upregulated in the CNS during both acute and chronic inflammation, and in neurodegenerative diseases (34; 60; 281) microglia tend to remain in M1 activation states (151; 173). The aberrant persistence of microglia in M1 activation states for long periods of time following CNS injury therefore may in part be due to inappropriate responses to anti-inflammatory signals. Impaired responses to the anti-inflammatory cytokine TGF- β has previously been associated with increased proinflammatory responses in ageing (264; 276).

TGF- β is a pleiotropic cytokine which has strong anti-inflammatory properties in both CNS (45) and non CNS tissues (148). In the CNS, TGF- β mediates microglial quiescence, inhibits release of proinflammatory cytokines, modulates microglial proliferation and is directly neuroprotective (45; 212). TGF- β signals by binding TGF- β receptor 2 (T β R2) which then activates TGF- β receptor 1 (T β R1) by phosphorylation. T β R1 activates the receptor associated Smad proteins, Smad2 and Smad3, by phosphorylation which then translocate into the nucleus to regulate gene transcription (182). TGF- β signaling is autoregulated by induction of the inhibitory Smad, Smad7, through inhibition of T β R1 mediated smad2/3 phosphorylation (43; 203; 314). Other negative regulators of TGF- β signaling include the E3 ligases Smurf 1 and 2 (126), Smad function inhibitors Ski and SnoN (167) and the TGF- β pseudoreceptor Bambi (315).

The TGF- β signaling pathway interacts with many other pathways in the cell providing a mechanism for modulation of the pathway by other signals (136; 248). Toll like receptors (TLRs) transduce signals from the inflammatory molecules that are produced after CNS injury (82). TLR signaling results in activation of microglia (82) producing effects that oppose TGF- β signaling. Several studies have shown that TGF- β signaling inhibits TLR4 activated pathways and other proinflammatory signals in microglia (54; 91; 136; 141) but little is known about how proinflammatory signals impact on the TGF- β signaling pathway. We have recently shown that the TLR4 agonist lipopolysaccharide (LPS), inhibits TGF- β signaling in primary microglia through reducing expression of T β R1, lessening Smad phosphorylation and ameliorating TGF- β mediated microglial cell death (194). Thus, prolongation of pro-inflammatory signals leading to chronic inflammation may be achieved at least in part through down regulation of anti-inflammatory pathways. Therefore, to understand better the mechanisms underlying chronic microglial activation we need to elucidate how the proinflammatory signals interact with and alter anti-inflammatory pathways in microglia.

The stimuli recognized by microglia, like other macrophages, include damage associated molecular patterns (DAMPs) (156; 226) and pathogen associated molecular patterns (PAMPs) (78). Examples of PAMPs include bacterial cell wall components like lipopolysaccharides and lipoteichoic acids (78). Examples of DAMPs include cellular components like ATP and aggregated products of aberrantly cleaved amyloid precursor proteins, amyloid β (A β) (156; 226) In Alzheimer's disease (AD), accumulated A β in the brain has been associated with neurodegeneration. Although it is not clear whether A β deposition is the initiator of the disease or a mediator of it, A β deposition activates

microglia and induces chronic neuroinflammation, which contributes to the disease pathogenesis (100). A β activates TLR4 however on microglial however, it is also recognized by several other scavenger receptors.

Neuroinflammation associated with infections of the CNS has also been linked with neurodegeneration (204). Group B streptococcus, a common cause of meningitis and activator of TLR2 in microglia, has can cause neurodegeneration (161). *Listeria monocytogenes* is also a common cause of neonatal meningitis and a TLR2 activator (268).

In this study, we determine whether other ligands for TLRs, A β and heat killed *listeria monocytogenes* (HKLM), also inhibit the TGF- β signaling pathway, and start to determine the mechanisms through which these effects are produced. We focus on the effect of A β oligomers as these contribute to the neuroinflammation found in Alzheimer's disease. Thus, the pathways through which microglial activation is regulated by A β will have important implications for chronic microglial activation in AD.

MATERIALS AND METHODS

Materials

TGF- β 1 was purchased from PeproTech (Rocky Hill, NJ, USA). The I κ B inhibitor Bay-11-7082, and LPS from *E. coli* 0111:B4 were purchased from Sigma-Aldrich (St Louis, MO, USA). A β 1-42 HFIP was purchased from r-Peptide (Bogart, GA, USA). The TBK1 inhibitor BX-795 and TLR2 agonist HKLM were purchased from Invivogen (San Diego, CA, USA). PD98059, an ERK1 inhibitor was purchased from EMD Millipore (Billerica, MA, USA). Primers were synthesized by Integrated DNA technologies (Coralville, Iowa, USA). Dulbecco's modified Eagle's medium (15-013-CV), Hanks' balanced salt solution (21-022-CV) and antibiotic-antimycotic solution (30-004-CL) were

purchased from Corning Cellgro (Manassas, VA, USA). Donor equine serum was purchased from Hyclone Thermo Scientific (Waltham, MA, USA) and Benchmark Fetal Bovine serum was purchased from Gemini Bio-Products (Sacramento, CA, USA). Complete Mini protease inhibitor cocktail tablets (04 693 124 001) were purchased from Roche (Nutley, NJ, USA). T β R1 antibodies (sc 398) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Primary microglia: All animal care and procedures were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University. Sasco/Sprague–Dawley rats (male and female) at age P1 were purchased from Charles River Laboratories (Frederick, MD, USA) and housed in the USU animal facility. Mixed glial cultures were made as previously described by Mitchell et al (2014). Brains were removed from the pups at P2, the cerebral hemispheres dissected out and placed in culture medium (Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 1% glutamax and 1% antibiotic-antimycotic) after their meninges were removed with forceps. Cortices were then triturated twice with a 10 mL pipette, followed by successive triturations with an 18G needle (3X), a 22G needle (3X), and finally a 25G needle (1X). The resulting suspension was filtered using a 70 μ m nylon mesh before pelleting at 168g for 10 min at 25°C. The cortical tissue homogenate was suspended in culture medium and seeded into T75 flasks at a density of half a cortex/flask. Cells were incubated in medium (Dulbecco's modified eagle's medium with 10% FBS, 1% glutamax and 1% streptomycin/penicillin) under standard conditions (37°C; 5% CO₂) and medium was replaced every 3 or 4 days. On day 14 the flasks were shaken for 1 hour at 200 rpm (MaxQ

2000 orbital shaker; Thermo Scientific, Rockford, IL, USA) to detach microglia. The microglia in the medium were collected and centrifuged at 671g for 5 min at room temperature followed by counting, resuspension and plating in 96 well plates at a density of 5×10^5 cells/well for qPCR, and the MTS assay. For cell proliferation assays and protein extraction, microglia were plated at a density of 3×10^6 cells/well in 6 well culture plates. The cells were grown for 24 hours before treatment.

BV2 cells: BV2 cells, between passages 8 and 20, were grown in 6 well plates containing DMEM supplemented with 10% normal horse serum, 1% glutamax and 1% streptomycin/ penicillin. The cells were plated at a density of 1.5×10^5 cells per well. After 24 hours of incubation, the cells were pulsed with EDU and used as positive control for EDU incorporation.

Oligomerization of A β peptides

Lyophilized A β (0.1mg) was dissolved in 10 μ L dimethyl sulfoxide and vortexed for 30 seconds. 90 μ L DMEM was added, and the solution was vortexed again for 30 seconds. The solution was then placed at 4 °C for 24 hours before use to oligomerization.

RNA isolation and Quantitative PCR

RNA was isolated using Trizol reagent according to manufacturer's instructions (Thermo Fisher Scientific, Carlsbad, CA), precipitated with isopropyl alcohol and resuspended in diethyl pyrocarbonate treated H₂O. RNA was quantified by UV spectrometry and its integrity was ascertained by gel electrophoresis. cDNA was synthesized from equal amounts of RNA with Superscript III reverse transcriptase (Thermo Fischer Scientific, Carlsbad, CA, USA) and SYBR Green QPCR performed in a CFX96 (Bio-Rad, Hercules, CA, USA). Target RNA expression levels were normalized to the

housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative changes in mRNA expression levels between control and treated samples were calculated by the delta threshold cycle ($\Delta\Delta C_t$) method (Susarla et al. 2011) using Bio-Rad CFX Manager 2.0 software. Primers used are listed in Table 1.

Western blots

Microglia were rinsed with ice cold PBS and lysed on ice with RIPA buffer before a brief sonication (10 seconds) on ice. Lysates were centrifuged to remove the insoluble fraction, and the supernatant collected for protein quantification. 2x loading buffer was added in equal volume with 15 μ g protein, boiled, and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes by semi-dry transfer (Transblot-turbo, Bio-rad), blocked in 5% milk/TBST for 1 hour followed by probing with primary antibody overnight. Membranes were rinsed in TBST, incubated with the appropriate secondary antisera linked to horseradish peroxidase for 1 hour and developed with chemiluminescence (Super Signal West Pico Chemiluminescent substrate, Thermo Fischer Scientific), before the image was acquired with the Bio-Rad Chemidoc Touch imaging system.

Cell counting

Microglia were plated in 96 well cell culture plates at 10⁵ cells/well the day before treatment. At the completion of time of treatment cells were washed with 1X PBS before fixing in 4% paraformaldehyde for 30 minutes. Cell nuclei were stained with 300nM 4,6-diamidino-2-phenylindole (DAPI) for 5 minutes. Images were obtained at 5X magnification using the Olympus microscope IX73. One field per well was acquired with

four wells per treatment group. All nuclei were counted in each image utilizing Image J software (National Institutes of Health, Bethesda, MD, USA).

MTS Assay

Primary microglia were plated in 96 well plates at a density of 5×10^5 cells/ well. Cells were then treated with A β with or without TGF β for 12 hours. 20 μ L of MTS reagent (Cell Titer 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) was then added to the cells in 100 μ L of medium for 1 hour. Cell viability was assayed using the H1 Synergy multimode microplate reader (BioTek) at an absorbance of 540nm.

EDU cell proliferation assay

Primary microglia or BV2 cells were plated in 6 well plates at a density of 1.5×10^5 cells/well. Primary microglia were treated with or without A β and EdU for 10 hours. Untreated BV2 cells treated with EdU were used as a positive control for proliferating cells. The cells were then gently scraped, fixed and processed with reagents from Click-IT EDU Alexa-Fluor 647 Flow Cytometry assay Kit (Thermo Fischer Scientific) according to manufacturer's instructions. EdU incorporation was determined using flow cytometry on the Acuri C6 flow cytometer (BD Bioscience, San Jose, CA, USA).

Statistical analysis

All data are expressed as mean $\pm$ SEM. Data were analyzed using Prism software (Graphpad, La Jolla, CA, USA). A minimum of four independent wells were assayed per treatment group and the effects were repeated in at least two independent experiments. A representative experiment is shown in all figures. For assessment of gene expression by

QPCR experiments, corrected expression was normalized to untreated samples and expressed as fold change in expression. Significant changes in reduced or induced gene expression relative to control wells was calculated by one way ANOVA with Dunnet's correction for multiple comparisons. Analysis of the effects of treatment on normalized T β R1 expression by western blot was also performed by one-way ANOVA with Sidak's correction for multiple comparisons. For assessment of the effect of different treatments on microglial cell number at different time points we performed a two way ANOVA.

RESULTS

T β R1 mRNA is rapidly reduced by different microglial activators

We have previously shown that expression of T β R1 mRNA is reduced in primary rat microglia following an 8-hour treatment with LPS (194). To determine the minimum time required for LPS signaling to reduce T β R1 mRNA expression we treated cells for different times with LPS and analyzed T β R1 mRNA expression by qPCR. LPS significantly reduced T β R1 mRNA within 1 hour of treatment, and the reduction was almost maximal by 2 hours (Figure 6A). LPS induced IL-1 β mRNA with similarly rapid kinetics (Figure 6B). We then sought to determine whether other activators of microglia had a similar effect on T β R1 gene expression. We treated primary microglia with increasing concentrations of either oligomerized A β , or HKLM, a freeze-dried heat-killed preparation of *Listeria monocytogenes* and TLR2 agonist. T β R1 mRNA was significantly reduced in a dose dependent manner in microglia treated with A β (Figure 6C) or HKLM (Figure 6E) for 6 hours with a maximum effect at 100nM A β and 10^7 cells HKLM. A β and HKLM also induced IL1 β mRNA with a similar concentration dependence (Figure 6D and 1F). To exclude the possibility that the observed effect of A β was due to LPS contamination

of the peptide, we repeated the A β treatment of microglia in the presence of the LPS blocker polymyxin B (PMX). Polymyxin B at 10 μ M completely blocked LPS (100ng/ml) mediated down regulation of T β R1 mRNA and prevented induction of IL1 β mRNA but did not block the A β effects on expression of either T β R1 or IL1 β mRNA (Figure 7A).

LPS and A β reduce TGF β mediated gene expression

Next, we assessed whether either LPS or A β treatment in microglia altered TGF- β regulated gene expression. T β R1 mRNA was induced approximately 4 fold and Smad6 mRNA approximately 10 fold by a 14-hour treatment with TGF- β (Figure 8). LPS or A β treatment alone reduced T β R1 and Smad6 mRNA expression. When microglia were treated with TGF- β in the presence of either A β or LPS, the TGF- β mediated induction of both T β R1 and Smad6 mRNA was completely abrogated (Figure 8). TGF- β mediated induction of T β R1 protein in primary microglia was also reduced after pretreatment with A β (Figure 9), replicating our previous finding with LPS (194).

A β treatment prevents TGF β mediated death of primary microglia

We previously showed that TGF- β treatment reduced the survival of primary microglia, but that addition of LPS prevented the TGF- β mediated reduction in microglial viability (194). We therefore sought to determine the effect of A β treatment on microglial survival in the absence or presence of TGF- β . Untreated primary microglial cells show a gradual decline in cell number in culture (Figure 10A). Treatment of microglia with TGF- β for 1 or 3 days significantly reduced the number of cells compared to untreated controls. TGF- β treatment did not reduce cell numbers in primary microglia treated with A β as compared to non-A β treated cells (Figure 10A).

Since we observed an increased number of microglia in A β treated wells compared to untreated wells (Figure 10A) we wanted to differentiate whether A β inhibited microglial cell death, or induced microglial cell proliferation. We therefore determined the amount of proliferation in microglia after A β treatment by quantifying EDU incorporation into DNA. We pulsed EDU for 10 hours but saw no EDU incorporation into primary microglial cells with or without A β treatment (Figure 10B). Therefore, A β did not induce microglial proliferation. However, in BV2 microglia cells, 10 hours EDU pulse resulted in the vast majority of BV2 cells incorporating EDU in this time frame (Figure 10B). We also assessed cell viability after a 12-hour treatment period using the MTS assay. Primary microglia treated with A β showed a significant increase in viability, which was not reduced in the presence of TGF- β (Figure 10C). However, in this shorter treatment time, TGF- β treatment alone did not alter microglial viability.

A β and LPS-mediated down regulation of T β R1 mRNA is dependent on the NF- κ B signaling pathway.

To start to determine the molecular mechanism through which LPS and A β act to reduce T β R1 mRNA, we treated microglia with different pharmacologic inhibitors of candidate signaling pathways before addition of either LPS or A β . Antagonism of TGF- β signaling has been shown to be mediated by activation of the MEK kinase (Kim *et al* (2011), by the IRF3 pathway (310) as well as through NF- κ B signaling (27). We therefore assessed the effect of inhibition of each of these pathways in LPS and A β activated microglia. Neither inhibition of the ERK pathway, with the MEK1 inhibitor PD98059, nor inhibition of the IRF3 pathway with BX795, a TBK1 inhibitor, prevented LPS-mediated down regulation of T β R1 mRNA (Figure 11E). However, inhibition of NF- κ B signaling

with Bay-11-7082 (BAY), an inhibitor of I κ B kinase partially prevented down regulation of T β R1 mRNA by A β (Figure 11A) and LPS (Figure 11C).

A β and LPS transiently induce the TGF β pathway inhibitors Bambi and SnoN but not Smad7

Several different genes are known negative regulators of TGF- β signaling. We therefore examined whether treatment of microglia with either LPS or A β led to induction of any of these inhibitors. We found that mRNA for Bambi, an inhibitory TGF- β pseudoreceptor (209), and SnoN, a Smad inhibitor (66), were transiently induced by treatment with either LPS or A β within 3 hours of treatment (Figure A,B,C,D). A β , but not LPS treatment led to downregulation of the inhibitory Smad, Smad7, with LPS treatment trending in this direction (Figure 12E,F).

DISCUSSION

Down regulation of anti-inflammatory signaling pathways is one mechanism through which inflammatory signals can prolong microglial activation. In this paper we show that A β in addition to LPS, downregulates anti-inflammatory TGF- β signaling in primary microglia, partially through a significant reduction in T β R1 mRNA and protein. We find that the reduction in TGF- β receptor mRNA is very rapid, initiating within an hour of treatment, and that the mechanism involves the NF- κ B signaling pathway. This results in reduced ability of the cell to transmit a TGF- β signal, and therefore may enhance inflammation. A β -mediated reduction in microglial T β R1 may therefore contribute to the chronic neuroinflammatory state in AD.

Our results show that A β and HKLM, in a similar manner to LPS, significantly reduce expression of mRNA encoding the signal transducing TGF- β receptor, T β R1 in

primary microglia (Figure 6). Given the key role of T β R1 in transducing TGF- β signaling, the reduction in T β R1 is a critical step in the ability of these microglial activators to antagonize TGF- β signaling. Indeed, the reduction in TGF- β signaling was highlighted by the total amelioration of TGF- β induction of two genes in the presence of either LPS or A β (Figure 8). That three different microglial activators produce a similar effect, indicates this may be a common mechanism through which microglial activators reduce anti-inflammatory signals to prolong the duration of their signals.

LPS and A β treatment resulted in a rapid reduction of T β R1 mRNA, which was evident within an hour of LPS treatment (Figure 6). The rapid response to TLR4 signaling suggests that the T β R1 mRNA is subject to specific stability regulation. While there are publications indicating that T β R1 mRNA is regulated by miRNAs (36; 307), the speed with which the RNA levels reduce suggest that RNA binding proteins may be involved with its regulation. Indeed the T β R1 gene has a very long 3'UTR providing many binding sites for AU rich binding proteins (47). It is possible that signals could cause either positive or negative regulation such that they modify an already bound RNA binding protein to remove it from stabilizing the T β R1 mRNA, or initiate protein binding to the 3'UTR to promote degradation. The NF- κ B dependent regulation of mRNA stability provides additional information to assist in the identification of such a protein.

We showed that inhibition of NF- κ B signaling, but not the ERK or IRF3 pathways, ameliorated the down regulation of T β R1 mRNA by A β and LPS (Figure 11). Inhibition of the TGF- β signaling via the NF- κ B pathway has been demonstrated in various cell types and contexts (27; 107) however our results show for the first time that NF- κ B pathway could antagonize TGF- β signaling by directly reducing T β R1 expression. In fibroblasts,

LPS inhibited TGF- β induced Smad signaling through NF- κ B dependent up regulation of Smad7 (27). Physiologic relevance of this mechanism was shown in patients with inflammatory bowel disease where Smad7 inhibition of TGF- β signaling sustained prolonged NF- κ B activity (197) and the use of the Smad7 antisense oligonucleotide, Mongersen, which suppressed colitis in patients via restoration of TGF- β signaling (12). In our microglial cultures however, we did not observe any induction in Smad7 mRNA with LPS or A β treatment suggesting that NF- κ B mediated inhibition is working through a different mediator (Figure 12C). However, we did find early but transient induction of mRNA encoding two other antagonists of TGF- β signaling Bambi and SnoN (Figure 12A,B,C,D). Although we did not explore further the implications of these gene changes, additional studies examining alterations in TGF- β antagonists and their effect of on microglial activation would be relevant to understanding the mechanisms underlying chronic microglial activation.

A β treatment increased microglial cell number and cell viability even in the presence of TGF- β (Figure 10A and 10C). We have previously shown that TGF- β induces microglial cell death (194) although we did not detect any significant reduction in cell viability after TGF- β treatment alone in the MTS assay (Figure 10C). This may be due to the shorter time of treatment. A β treatment was able to counteract any effect of TGF- β on cell numbers after 1 or 3 days of treatment (Figure 10A). However, we did not observe any proliferation of primary microglia with A β oligomer treatment (Figure 10B) suggesting that A β effects on microglia are not due to increased proliferation but rather to improved cell viability. It has previously been shown that LPS improves cell viability in dexamethasone treated peritoneal macrophages (111) and TGF- β treated FaO rat hepatoma

cells (165). The increased viability of primary microglia in the presence of activators may provide an explanation for persistent microglial activation during chronic neuroinflammation in spite of increased anti-inflammatory mediators. Indeed, it has been shown that the number of microglia are increased in TG2576 transgenic mice a mouse model for Alzheimer's disease with elevated A β levels (175; 208).

There are bi-directional interactions between proinflammatory signals and the anti-inflammatory TGF- β pathway. In this study we have focused on inhibition of TGF- β signaling by microglial activators. However, several studies have shown that TGF- β inhibits the pro-inflammatory TLR pathway via diverse mechanisms (54; 91; 160; 202). For example, TGF- β exerts anti-inflammatory action by inducing expression of Smad6, which directly inhibits TLR4 signaling through its interaction with Pellino-1(54) or MyD88 (160). Indeed, a Smad6 derived peptide, Smaducin6, was developed for the treatment of inflammation in sepsis because of its ability to inhibit TLR4 pathways (159). We have shown that Smad6 induction by TGF- β is strongly reduced by A β and LPS (Fig. 2). This reduction of a TGF- β dependent anti-inflammatory molecule provides another mechanism through which these microglial activators prolong microglial activation. Augmentation of Smad6 may thus represent a mechanism to enhance TGF- β anti-inflammatory effects without excessively increasing the amount of TGF- β in the CNS.

Chronic microglial activation and persistent neuroinflammation is a well known feature of AD brains (193; 249). However, no previous study has looked at the possibility of impairment of TGF- β signaling in microglia as a contributing factor to this chronic inflammation. Inhibition of anti-inflammatory pathways by proinflammatory mediators have, however, been previously associated with chronic microglial activation. Masocha

(2009) showed that reduction in the anti-inflammatory CD200 in association with increased microglial activation markers was observed for up to one year after intraperitoneal administration of LPS in mice (181). It would be interesting to determine whether or not microglial TGF- β receptor expression is lower in the brains of patients with AD or other neurodegenerative diseases.

The role of TGF- β in AD brains is quite complex (276). Elevated TGF- β levels have been detected in CSF and serum of AD patients (43; 323). However, impairment of TGF- β signaling in AD brains due to reduced neuronal expression of T β R2 (262) and impaired smad 3 action in microglia has been suggested (276). TGF- β directly induces expression of the APP gene in human astrocytes (9) implicating TGF- β in the pathophysiology of AD. However, in mouse models of AD, interfering with neuronal TGF- β signaling by pharmacologic or genetic means led to increased neurodegeneration and susceptibility to A β effects (38; 262) due to reduction in the neuroprotective effects of TGF- β . Additionally, TGF- β has anti-amyloidogenic roles as TGF- β promotes the microglial mediated phagocytosis of A β leading to its clearance from the CNS parenchyma (302). Thus, A β mediated impairment of TGF- β signaling in microglia would impact on the ability of the microglia to clear plaques allowing for a greater detrimental effect of A β on neurons. Thus, overall, TGF- β signaling in microglia and in neurons should reduce the toxic effects of A β on these cells, yet A β effectively counteracts these beneficial effects, further promoting a vicious cycle.

CONCLUSION

In conclusion, we have shown *in vitro* that TGF- β signaling is compromised in microglia activated with the TLR ligands, A β , LPS and HKLM. The TGF- β pathway is an important anti-inflammatory pathway which when compromised predisposes to unregulated inflammation that contributes to neurodegeneration. We show that the NF- κ B pathway plays a role in inhibition of TGF- β signaling in microglia (Figure 13) however, further studies would be needed to assess more detail mechanisms and the role of other proinflammatory pathways. Strategies that augment the effects of TGF- β in both neurons and microglia in patients with Alzheimer's disease or other neurodegenerative disorders may therefore be of benefit in ameliorating these pathologies. A thorough understanding of regulation of cross-talk between proinflammatory and anti-inflammatory processes that occur in microglia could help in development of therapies that restore normal CNS homeostasis.

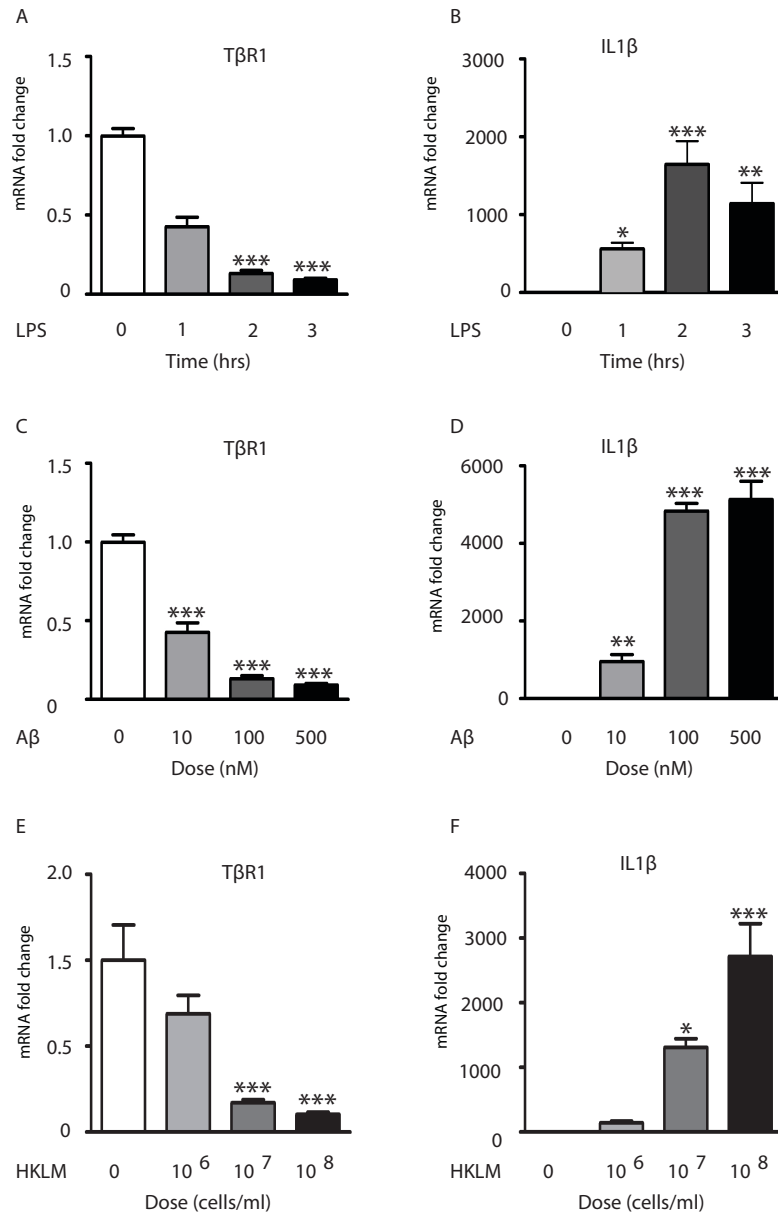


Figure 6. LPS, Aβ and HKLM reduce TβR1 mRNA in primary microglia.

Primary microglia were treated with LPS, Aβ or HKLM for the indicated times. RNA was extracted and qPCR targeting TβR1 and IL1β performed. Graphs indicate reexpression of (A,C,E) TβR1 or (B,D,F) IL1β mRNA normalized to GAPDH. (Mean ± s.e.m., n=4 (***)p<0.001, (**)p<0.01, (*)p<0.05).

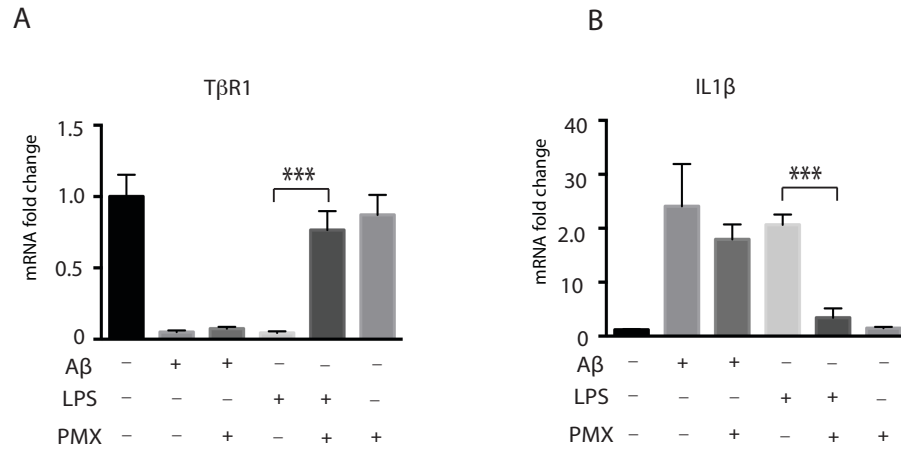


Figure 7. Polymyxin B inhibits LPS but not Aβ mediated down regulation of TβR1 in rat primary microglia

Primary microglia were pretreated with Polymyxin B (PMX) (10μM) for 1 hour followed by LPS or Aβ for 6 hours. LPS but not Aβ mediated reduction in TβR1 mRNA was significantly inhibited by PMX pretreatment (A). PMX also significantly inhibited LPS but not Aβ mediated

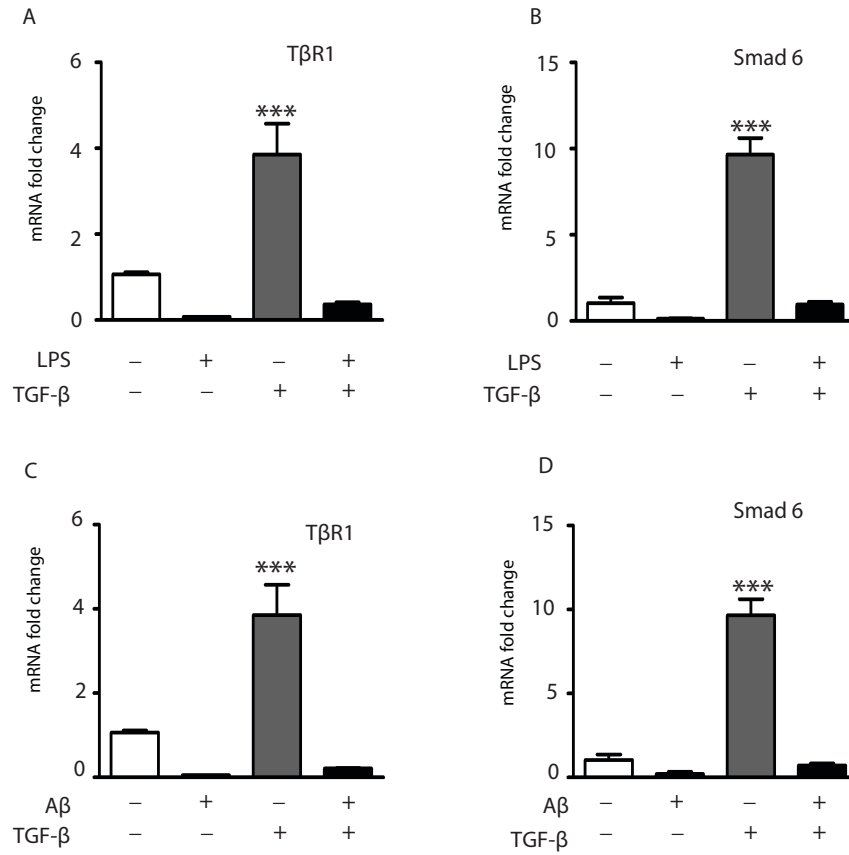


Figure 8. LPS and Aβ reduce TGF-β induction of TβR1 and Smad6 mRNA.

Primary microglia were pretreated with LPS (100 ng/ml) (A,B) or Aβ (100 ng/ml) (C,D) for 16 hours before the addition of TGF-β for 6 hours. RNA was extracted and qPCR targeting Smad6 and TβR1 performed. Both Aβ and LPS significantly inhibited TGF-β dependent induction of TβR1 (A,C) and Smad6 mRNA (B,D) respectively. (Mean ± s.e.m, n=4, ***p<0.001).

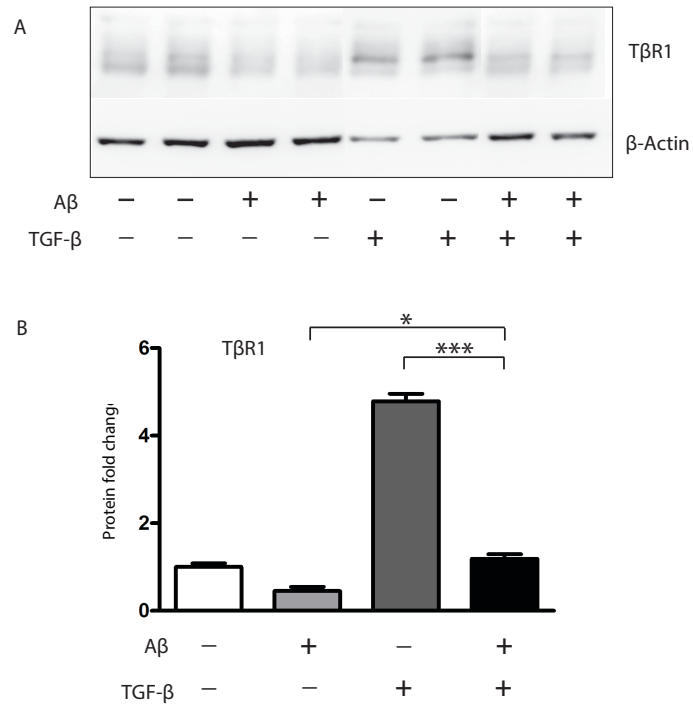


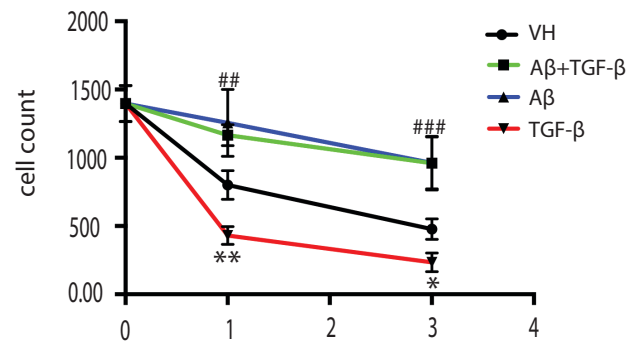
Figure 9. Aβ reduces TβR1 expression in rat primary microglia.

(A) Western blot of lysates from primary microglia after treatment with Aβ (100nM) for 14 hours followed by TGFβ (1ng/ml) for 10 hours. (B) Quantification of expression of TβR1 from the western blots, normalized to β-actin. (Mean ± s.e.m, n=3 ***p<0.001, *p<0.05).

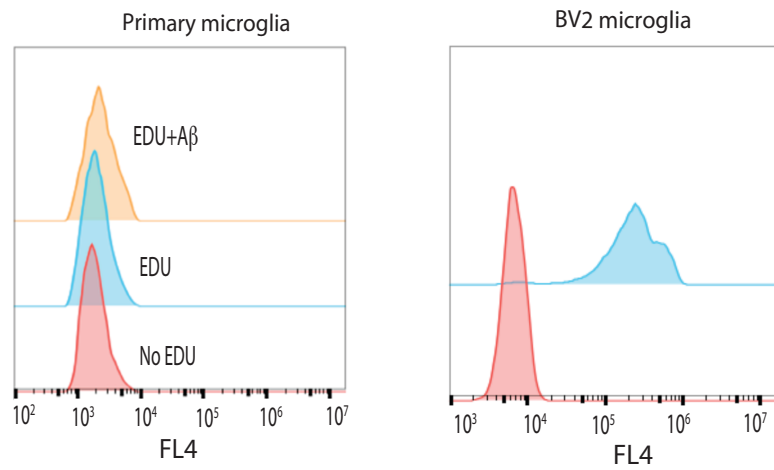
Figure 10. A β prevents TGF β induced death of primary microglia.

(A) Primary microglia were treated with (100nM) and/or TGF- β (1ng/ml) as indicated for 1 or 3 days before fixation, staining and imaging. Counts are the average number of cells/field from each of 4 wells. The microglial cell population decreased with time in culture. TGF- β treatment however significantly increased cell loss compared to vehicle treatment at both one (**p<0.01) and three days (*p<0.05). A β treatment did not only prevent the normal decline in microglia cells, it significantly prevented TGF- β mediated reduction in microglial cell population at both one day (##p<0.01) and 3 days (###p<0.001) of treatment. (B) FACS analysis of cells to determine EDU incorporation after a 10 hour EDU incubation. Primary microglia did not proliferate after A β treatment. However, BV2 cells did incorporate EDU indicating proliferation. (C). Primary microglia were treated with A β (100ng/ml) and/or TGF- β (1ng/ml) as indicated for 12 hours. Cell viability was assessed by MTS assay. Cell viability was significantly increased in wells treated with A β (**p<0.01) or A β and TGF- β (*p<0.05). (Mean $\pm$ s.e.m, n=4).

A



B



C

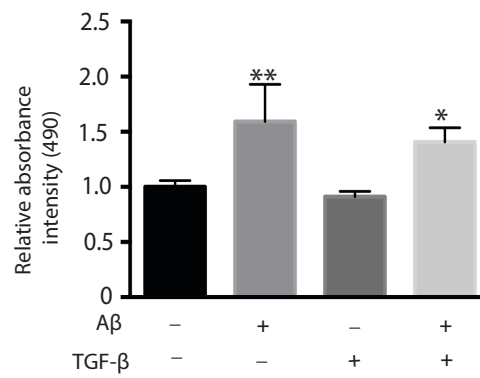
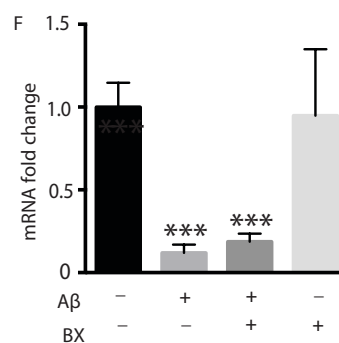
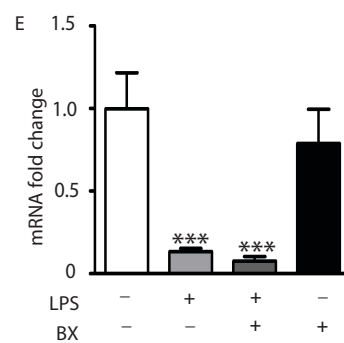
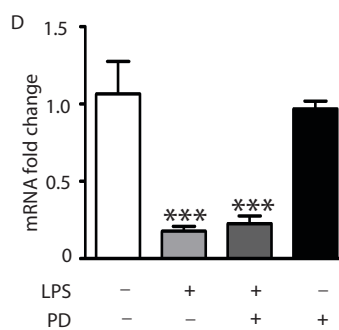
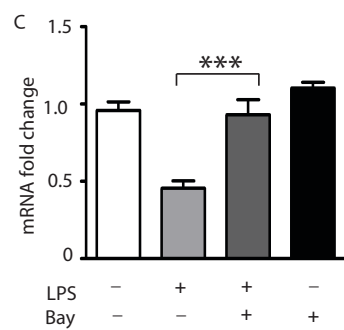
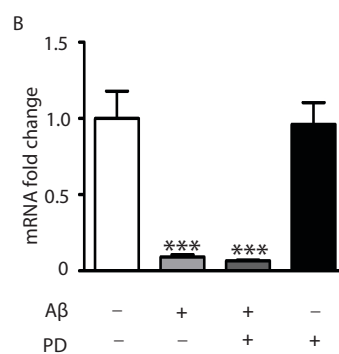
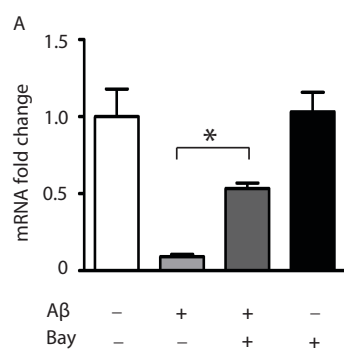
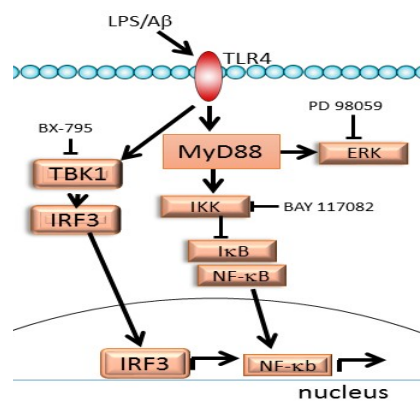


Figure 11. Inhibition of the NF- κ B pathway but not the MAP Kinase or IRF3 pathway reduces A β and LPS mediated down regulation of T β R1.

Graphs show mRNA expression of T β R1 in primary microglia after different treatments (Schematic above graphs). Primary microglia treated with LPS (100ng/ml) (C,D,E) or A β (100nM)(ABF) alone for 6 hours significantly reduced T β R1 mRNA (**p<0.001). Pretreatment with the MEK1 inhibitor PD98059- 20mM (PD) or TBK1 inhibitor BX-795- 1 μ M, 30 minutes before LPS or A β treatment did not significantly increase T β R1 mRNA (B,D,E,F). However pretreatment with the I κ B inhibitor (BAY-11-7082- 2 μ M) for 30 minutes significantly increased T β R1 mRNA reduced by A β (*p<0.05) (A) and LPS (**p<0.001) (C). (Mean $\pm$ sem, n=4).



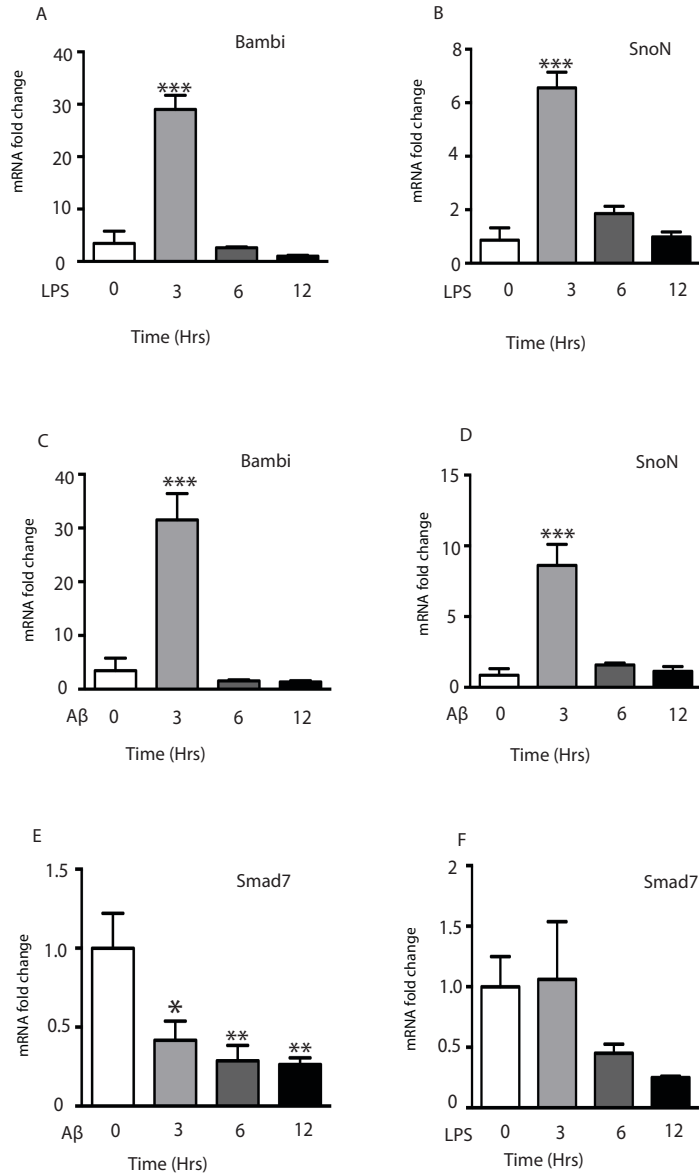


Figure 12. Changes in gene expression of inhibitors of TGF- β signaling.

Primary microglia were treated with LPS (100ng/ml) or A β (100nM) for 6 hours followed by RNA extraction and qPCR targeting genes encoding inhibitors of TGF- β signaling pathway: Bambi, SnoN and Smad7. LPS (A,B) and A β (C,D) treatments transiently induced Bambi and SnoN mRNA. However, neither LPS (E), nor A β induced Smad7 mRNA in rat primary microglia (E, F) (Mean $\pm$ sem, n=3; ***p<0.001, **p<0.01, *p<0.05).

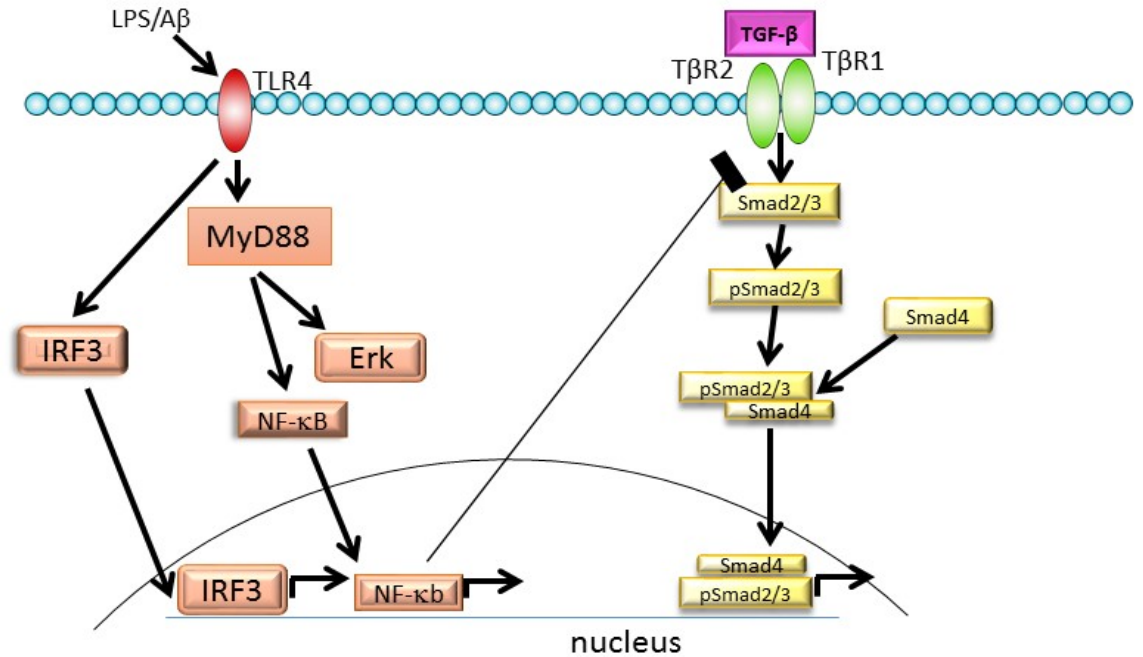


Figure 13. Schematic illustration of NF- κ B dependent inhibition of TGF- β signaling

Primary microglia were treated with LPS (100ng/ml) or A β (100nM) for 6 hours. Activation of TLR4 by LPS or A β leads to the activation of IRF3 or MyD88 pathways. MyD88 activates Erk and NF- κ B pathways. Our data shows that NF- κ B pathway activation mediates downregulation of T β R1 in the TGF- β signaling pathway.

CHAPTER 3: Telmisartan reduces LPS mediated inflammation and induces autophagy and death in microglia

Kwame Ofori Affram, Zachary Janatpour, Sonia Villapol, Aviva Jane Symes.

ABSTRACT

Chronic neuroinflammation mediated by persistent microglial activation is strongly associated with neurodegeneration. Reducing microglial activation as well as the population of activated microglia could therefore be of benefit in neurodegenerative disorders. Angiotensin receptor blockers (ARBs) have prominent anti-inflammatory activity in some cell types, and have been proposed as potential treatments for neurodegenerative diseases and neurotrauma. Although all ARBs act as antagonists at the angiotensin II receptor I, some ARBs also activate or inhibit other signaling pathways allowing for a multi modal mechanism of action. The mechanism of anti-inflammatory action of the ARBs has not been thoroughly investigated although recently the ARB telmisartan was shown to reduce microglial activation via the AMPK. In this study we compared the ARBs for their ability to reduce LPS stimulation of primary microglia or BV2 cells. We find that telmisartan showed the greatest efficacy to reduce LPS stimulated reactive oxygen species and nitric oxide production in primary microglia and BV2 microglial cells. Telmisartan, but not the other ARBs dose dependently induced AMPK in BV2 cells and reduced phosphorylation of the downstream kinase mTOR. Telmisartan's reduction of LPS stimulated reactive oxygen species was partially dependent on AMPK activity as the AMPK inhibitor partially attenuated this effect. Telmisartan also induced markers of autophagy in microglia and was the only ARB that reduced microglial viability. Telmisartan's toxicity to microglia was partially ameliorated by an inhibitor of autophagy

and a pan-caspase inhibitor. We conclude that telmisartan is able to activate AMPK and reduce mTOR activation and hence induce autophagy in microglia. Induction of autophagy has been shown to reduce inflammasome activation and is therefore a potential anti-inflammatory mechanism through which telmisartan acts in microglia. Reduction of microglial viability through multiple pathways also serves as an alternative anti-inflammatory mechanism to reduce the activated microglial population.

INTRODUCTION

Inflammation within the central nervous system is an integral feature of neurodegenerative diseases as well as neurotrauma. Although this inflammation may initially be beneficial, chronic neuroinflammation can escalate or worsen the neuropathology and hence disease progression. Indeed, chronic neuroinflammation has been strongly correlated with neurodegeneration (118; 173; 242). Drugs that ameliorate neuroinflammation present a reasonable therapeutic option for neurodegeneration and neurotrauma (145; 242).

Microglia are an important cellular contributor to the development and modulation of inflammation in the central nervous system (137; 145). Exposure of microglia to signals from pathogens or damaged cellular components, activates microglia to a pro-inflammatory phenotype where they increase their production of pro-inflammatory cytokines such as IL-1 β and TNF α , and upregulate production of NO and reactive oxygen species (ROS). Although this M1-like phenotype can be useful for host defense and phagocytosis, the products from these activated microglia are harmful to neurons, and prolonged M1-like responses can lead to a damaging hyperinflammatory state (49; 247). Microglial activation following some CNS insults may persist in this M1-like

proinflammatory state leading to neuronal injury and neurodegeneration (170; 173). However, anti-inflammatory signals can alter the microglial phenotype to different M2-like states that either enhance phagocytic activity, or promote tissue repair and remodeling (247). Although this polar definition of microglial phenotype is increasingly disputed as many microglia express markers of both states (220), the retention of a more pro-inflammatory phenotype is prevalent in microglia after traumatic brain injury or in neurodegenerative diseases (10). Reduction of this pro-inflammatory state, and/or shifting microglial phenotype to a less inflammatory state is therefore an important therapeutic target for prevention and mitigation of neurodegeneration.

Angiotensin receptor blockers (ARBs) are FDA approved drugs used extensively in the treatment of cardiovascular disorders including hypertension, heart failure and diabetic nephropathy (16; 131; 134). ARBs act as antagonists at the angiotensin II (Ang II) type I receptor (AT1R) to block Ang II mediated increases in blood pressure through vasoconstriction or increased release of aldosterone from the zona glomerulosa of the adrenal gland (16; 134). Besides their antihypertensive effects, ARBs also have anti-inflammatory effects that assist their beneficial effects in the cardiovascular system. Signaling through AT1R leads to activation of some proinflammatory pathways including the NF- κ B pathway, NADPH oxidase and the production of ROS and proinflammatory mediators (134; 201). ARB's anti-inflammatory effects are mediated by both AT1R dependent and independent mechanisms (228; 274).

Although the RAS was initially described mainly outside the brain, more recent research has revealed the presence of all components of RAS within the CNS (127; 150; 195; 233). These findings have opened up new avenues for therapeutic options for ARBs.

A growing number of research studies have demonstrated neuroprotective effects of ARBs in preclinical models of Alzheimer's disease, Parkinson's disease, stroke and traumatic brain injury (18; 228; 272; 273). In addition to their neuroprotective effects, ARBs have also been shown to have anxiolytic and anti-depressive properties, and to enhance cognitive function- effects that are probably mediated at least in part by regulation of AT1R signaling in the brain. Many of the neuroprotective effects of ARBs have been attributed to their anti-inflammatory effects in CNS tissues both through AT1R dependent and independent mechanisms.

There are currently 8 FDA approved ARBs. Although all these drugs act as antagonists at the AT1R, they vary in their ability to modulate non AT1R signaling pathways. Losartan, telmisartan, candesartan and irbesartan act as partial agonists at the anti-inflammatory PPAR γ receptor (76). Telmisartan activates PPAR γ more potently than all the other ARBs (20). Until recently it was assumed that some of the anti-inflammatory effects of ARBs were mediated through activation of PPAR γ (77). However, recently telmisartan has been shown to activate AMPK in a variety of cell types, including microglia. The anti-inflammatory actions of telmisartan in microglia were mediated by AMPK activation (312). We have shown that both candesartan and telmisartan have efficacy in improving recovery in a mouse model of TBI, including reducing the amount of microglial activation after injury (272) and its therapeutic potential in disorders with an inflammatory component.

MATERIALS AND METHODS

Materials

The AMPK inhibitor, Compound C and the PPAR γ inhibitor T0070907 were purchased from Sigma-Aldrich (St Louis, MO, USA). The CYTO-ID autophagy assay kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Primers were synthesized by Integrated DNA technologies (Coralville, Iowa, USA). Dulbecco's modified Eagle's medium (15-013-CV), Hanks' balanced salt solution (21-022-CV) and antibiotic-antimycotic solution (30-004-CL) were purchased from Corning Cellgro (Manassas, VA, USA). Donor equine serum was purchased from Hyclone Thermo Scientific (Waltham, MA, USA) and Benchmark Fetal Bovine serum was purchased from Gemini Bio-Products (Sacramento, CA, USA). Complete Mini protease inhibitor cocktail tablets (04 693 124 001) were purchased from Roche (Nutley, NJ, USA). The LC3B antibody (NB100-2220) was purchased from Novus Biologicals (Littleton, CO). Antisera recognizing AMPK (2532S), p-AMPK (2535S), mTOR (2972S) and p-mTOR (2971S) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti- β -Actin (A1978), LPS, telmisartan, candesartan, losartan, irbsartan, azilsartan, valsartan and eprosartan were purchased from Sigma-Aldrich (St Louis, MO, USA). Candesartan (AstraZeneca, CV-11974) was purchased from astra-zeneca (Sweden). Faststart universal SYBER Green was purchased from Roche (Indianapolis, IN).

Cell culture

Primary microglia

All animal care and procedures were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University. One-day-old Sasco/Sprague–

Dawley rats (male and female) were purchased from Charles River Laboratories (Frederick, MD, USA). Mixed glial cultures were made as previously described by Mitchell et al (2014). Briefly, brains were removed from the pups on P2, the meninges were removed, and the cortices dissected into culture medium (Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 1% glutamax and 1% antibiotic-antimycotic). Tissue was triturated twice with a 10 mL pipette, followed by successive triturations with an 18G needle (3X), a 22G needle (3X), and finally a 25G needle (1X). The resulting suspension was filtered using a 70µm nylon mesh before pelleting at 168g for 10 min at 25°C. The cortical tissue homogenate was suspended in culture medium and seeded into T75 flasks at a density of half a cortex/flask. Cells were incubated in medium (Dulbecco's modified eagle's medium with 10% FBS, 1% glutamax and 1% streptomycin/penicillin) under standard conditions (37°C; 5% CO₂) and medium was replaced every 3 or 4 days. On day 14 the flasks were placed on an orbital shaker (MaxQ 2000 orbital shaker; Thermo Scientific, Rockford, IL, USA) and shaken for 1 hour at 200 rpm to detach microglia. The medium containing microglia was collected and centrifuged at 671g for 5 min at room temperature followed by counting, resuspension and plating in 96 well plates at a density of 5×10^5 cells/well for qPCR, and analysis of NO and ROS. 12 hours prior to treatment, the media was replaced with media containing 2.5% FBS.

BV2 cell culture

BV2 microglia between passages of 6 and 20 were used. Cells were plated at 1.5×10^5 cells/ well in 96 well plates at 200µl/well and 2.5×10^5 cells/ well in 6 well plates with 2ml media /well for 24 hours. Culture medium was 10% normal horse serum in

DMEM supplemented with 1X glutamate. Cells were serum starved overnight before treatment.

RNA isolation and Quantitative PCR

RNA was isolated using Trizol reagent according to manufacturer's instructions (Thermo Fisher Scientific, Carlsbad, CA). Briefly, treated cells were washed once with 1X PBS followed by addition of 500 μ L of Trizol. Phase separation was done by addition of chloroform followed by centrifugation at 14000g for 15 minutes. The aqueous phase was carefully transferred into new 1.5ml eppendorf tubes and the RNA precipitated with isopropyl alcohol. RNA was quantified by UV spectrometry and its integrity ascertained by gel electrophoresis. cDNA was synthesized from equal amounts of RNA with Superscript III reverse transcriptase (Thermo Fischer Scientific, Carlsbad, CA, USA). SYBR Green qPCR was performed in a CFX96 (Bio-Rad, Hercules, CA, USA). Target gene expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the delta threshold cycle ($\Delta\Delta C_t$) method (Susarla et al. 2011) and analyzed with Bio-Rad CFX Manager 2.0 software.

Western blots

Cells were washed with ice-cold PBS followed by lysis with RIPA buffer for 10 minutes at 4°C. Lysates were centrifuged to remove the insoluble fraction, and the supernatant collected for protein quantification by BCA assay. 6X loading buffer was added and the samples denatured at 100°C for 5 minutes. 15 μ g protein was separated by SDS PAGE. Proteins were transferred onto membranes (PVDF- LC3B, nitrocellulose—all others) by semi-dry transfer (Transblot-turbo, BioRad). Membranes were blocked in 5% milk/TBST for 1 hour followed by probing with primary antibody overnight. Membranes

were rinsed in TBST, incubated with the appropriate secondary antisera linked to horseradish peroxidase for 1 hour and developed with chemiluminescence (Super Signal West Pico Chemiluminescent substrate, Thermo Fischer Scientific). Images were acquired and analyzed with a Chemidoc Touch imaging system (BioRad).

Autophagy assay

BV2 microglia were plated in 96 well plates and treated as required. Cells were analyzed for autophagy using the CYTO-ID assay (Enzo, New York). Briefly, medium was removed after required treatment and cells washed in 1X PBS. CYTO-ID reagent was prepared using 1:1000 dilution of CYTO-ID reagent and Hoechst 33342 nuclear stain. 100 μ L diluted CYTO-ID reagent was added to each well and incubated for 30 minutes at 37°C. The dual detection reagent was removed and the cells were washed once with PBS. Plates were read in the Synergy H1 fluorescent plate reader (Biotek) with excitation wavelength of 480nm and emission of 530nm for CYTO-ID reagent and excitation of 340nm excitation and 480nm emission for hoescht dye (DAPI filter). Fluorescence intensity for CYTO-ID was normalized to that of Hoechst for each well.

Reactive oxygen species (ROS) Assay

ROS was assessed by H2DCFDA assay according to manufacturer's instructions (Thermo Fischer Scientific, Waltham, MA, USA). Briefly, 20 μ M H2DCFDA solution was diluted in HBSS. 100 μ L H2DCFDA solution was added to each well and incubated for 30 minutes at 37 degrees. Fluorescence intensity was determined with the Synergy H1 plate reader at excitation wavelength of 470nm and emission wavelength of 530nm.

Griess Assay

Primary microglia or BV2 cells were plated in 96 well plates and treated as indicated. 50µl conditioned medium was removed, and combined with 50µl Griess assay reagent in a new plate (Signa-Aldrich, St. Louis, MO, USA). The reaction was incubated in the dark for 30 minutes at room temperature. The amount of NO generated was determined by reading the absorbance at 540nm in the Synergy H1 plate reader.

LDH Assay

After treatment, 50µl of culture medium was pipetted into a separate 96 well plate followed by addition of an equal volume of LDH reagent (Promega, Madison, WI, USA). Reactions were incubated for 30 minutes in the dark at room temperature before addition of stop solution. Absorbance at 490nm was determined in the synergy H1 plate reader.

MTS cell proliferation assay

Primary microglia or BV2 cells were plated in 96 well plates and treated as required. To assess cell viability, 20µL MTS reagent (Promega, Madison, WI, USA) was added to cells in 100µl of culture medium and incubated for 1 hour followed by reading of absorbance in the Synergy H1 plate reader at 540nm.

Trypan blue exclusion

Cells were grown in 6 well plates and treated as required. After treatment, the cells were washed with 1xPBS, trypsinized and transferred into 15 ml tubes. The cells were centrifuged for 2 minutes to remove trypsin followed by resuspension in PBS. 20µl cell suspension was combined with 20µl Trypan blue (0.4%) and exclusion was assessed using the Biorad TC20 automated cell counter.

Mitochondrial membrane potential assay

BV2 cells were plated in 6 well plates or 96 well plates as required. The cells were then treated with telmisartan for 6 hours followed by addition of TMRE (ThermoFischer Scientific) to 1 μ M concentration for 20 minutes. Cells from 6 well plates were harvested by trypsinization for flow cytometry in Acuri C6 flow cytometer (BD Bioscience, San Jose, CA, USA). Cells treated in 96 well plates were read on plate reader (Synergy H1 Hybrid plate reader, BioTek Instruments) and fluorescent images taken afterwards using Olympus microscope IX73.

Statistical analysis

All data are expressed as mean $\pm$ SEM. Data were analyzed using Prism software (Graphpad, La Jolla, CA, USA). A minimum of four independent wells was assayed in each treatment group and the effects were repeated in at least two independent experiments. A representative experiment is shown in all figures. For assessment of gene expression by qPCR experiments, corrected expression was normalized to untreated samples and expressed as fold change in expression. Significant changes in reduced or induced gene expression relative to control wells was calculated by one way ANOVA with Dunnet's correction for multiple comparisons.

RESULTS

Telmisartan counteracts LPS mediated inflammatory changes more strongly than other angiotensin receptor blockers.

We have previously shown that telmisartan and candesartan have anti-inflammatory action in a mouse model of TBI. However, it was not known whether other ARBs share this property. We therefore compared all 8 FDA approved ARBs for their

ability to reduce LPS stimulated nitric oxide and reactive oxygen production in rat primary microglia or in the mouse microglial BV2 cell line. 20 μ M telmisartan reduced LPS induced NO to a greater extent than 100 μ M of the other ARBs in either primary microglia (Figure 14A) or BV2 cells (Figure 14C). Telmisartan dose dependently reduced NO release. In primary microglia this effect was detectable with 1 μ M telmisartan, increasing up to 50 μ M telmisartan (Figure 14B). In BV2 cells 10 μ M telmisartan was sufficient to significantly reduce NO production by LPS, and 20 μ M telmisartan reduced NO production to levels produced in unstimulated cells (Figure 14D). LPS induced ROS generation in BV2 cells was similarly inhibited by 20 μ M telmisartan (Figure 14E,F). However, irbesartan and losartan also reduced LPS stimulated ROS production in BV2 cells (Figure 14E). Telmisartan also dose dependently reduced LPS mediated induction of iNOS and IL-1 β mRNA in primary microglia and BV2 cells (Figure 15). Thus, telmisartan has specific anti-inflammatory action in microglial cells that is not shared by other ARBs.

Angiotensin II did not induce proinflammatory genes in microglia

As a first step to determining the mechanism through which telmisartan reduces the production of anti-inflammatory mediators in microglia we determined whether Angiotensin II had pro-inflammatory action in these cells. Angiotensin II treatment of primary microglia did not induce either IL-1 β or TNF α mRNA, in contrast to the effects of LPS treatment (Figure 16A,B). If telmisartan's anti-inflammatory actions were mediated by blocking Angiotensin II signaling through the AT1 receptor, then we would expect Angiotensin II to have pro-inflammatory effects. The absence of these effects suggests that telmisartan is not acting through its effects on the AT1 receptor.

Telmisartan activates the AMPK/mTOR pathway

Telmisartan has recently been shown to activate AMPK pathway in microglia but the ability of other ARBs to activate AMPK was not known (312). We therefore compared the ability of Telmisartan and other ARBs to induce AMPK phosphorylation in BV2 cells. As previously reported 10 μ M telmisartan induced AMPK phosphorylation in BV2 cells, but candesartan, valsartan, irbesartan and olmesartan did not significantly alter AMPK phosphorylation at this dose (Figure 17A). The activation of AMPK by telmisartan was dose dependent (Figure 17B). Activated AMPK results in inhibition of mTOR phosphorylation (163). We therefore assessed whether telmisartan treatment led to reduce mTOR phosphorylation in BV2 cells. Telmisartan did indeed dose dependently reduce the amount of phosphorylated mTOR detected (Figure 17C).

Telmisartan's anti-inflammatory activity is partially inhibited by compound C

Some ARBs, in addition to acting as antagonists at the AT₁R, also act as partial agonists at PPAR γ (76). As activation of PPAR has anti-inflammatory activity in microglia (22), it was assumed that some anti-inflammatory activity may be mediated by PPAR γ effects. Indeed, telmisartan is the strongest partial PPAR γ agonist of the FDA approved ARBs (20; 76). To determine if PPAR γ may mediate the anti-inflammatory effects of telmisartan, we treated cells with a PPAR γ antagonist (T0070907). However, pre-treatment of microglia with two different concentrations of T007907 did not block telmisartan's ability to reduce LPS-mediated production of ROS or nitric oxide (Figure 18A,C) suggesting that telmisartan's activation of PPAR γ was not responsible for its anti-inflammatory activity.

To determine whether AMPK activity was responsible for the telmisartan's anti-inflammatory effects we pretreated BV2 cells with the AMPK inhibitor Compound C (CC) before adding telmisartan and then LPS. Inhibition of AMPK with compound C partially inhibited telmisartan's ability to reduce LPS generated ROS production (Figure 18B) but did not prevent telmisartan's ability to reduce LPS mediated NO production (Figure 18D). These data suggest that telmisartan anti-inflammatory ability involves the activation of AMPK but not PPAR γ .

Telmisartan induces autophagy in BV2 microglia

As AMPK activation with associated inhibition of mTOR is associated with induction of autophagy, we next determined whether telmisartan treatment resulted in increased autophagy in BV2 cells. We examined the ration of LC3BII to LC3BI, a marker of initiation of autophagosome and autolysosome formation (189). Ten hours of telmisartan treatment led to an increase in the LC3BII/1 ratio (Figure 19A). Co-treatment with chloroquine, a drug that blocks autophagic flux and therefore allows accumulation of autophagosomes, enhanced this LC3BII/1 ratio (Figure 19A). Dose-dependent telmisartan induced autophagy was also detected with the autophagy detection kit, cytoID (Figure 19C).

Telmisartan dose dependently reduce viability of microglial cells

Telmisartan has previously been shown to induce apoptosis in adult T-cell leukemia cells (147) and human endometrial cancer cells (146). The ability of ARBs to alter microglial viability is not known. We determined whether any of the eight ARBs altered microglial viability. Telmisartan was unique in its ability to increase LDH release from either primary microglial cultures or BV2 microglial cells (Figure 20A,C).

Telmisartan dose dependently induced LDH release in both cell types with 10 μ M telmisartan producing a significant induction of LDH release (Figure 20B,D). Telmisartan's effect of reducing BV2 microglial viability was confirmed by both the MTS cell proliferation assay (Figure 20E) and trypan blue exclusion (Figure 20F).

Inhibition of autophagy and apoptosis reduced telmisartan induced cell death in BV2 cells

Telmisartan has been shown to induce apoptosis in T-cell leukemia cells and vascular smooth muscle cells (163). To determine the mechanism of microglial cell death due to telmisartan we pretreated primary microglia with increasing doses of the autophagy inhibitor 3-MA or apoptosis inhibitor Z-VAD-FMK (20 μ M) prior to telmisartan (50 μ M) treatment. Telmisartan mediated LDH release was almost completely inhibited by Z-VAD FMK (15B) and partially inhibited with 2.5 μ M 3-MA (15A). These data indicate that telmisartan induces apoptosis in microglia and autophagy also accounts partially for some death inducing effects of telmisartan.

Telmisartan increases mitochondrial membrane potential in BV2 cells

One mechanism through which AMPK can be activated leading to induction of autophagy is through inhibition of the mitochondrial function leading to an increase in cytoplasmic AMP (275). Also reduction in mitochondrial membrane polarization can induce apoptosis (282). We therefore assessed whether telmisartan treatment results in alteration of the mitochondrial membrane potential in BV2 cells using a dye tetramethylrhodamine, ethyl ester (TMRE) that only accumulates in active mitochondria, according to their membrane potential. Surprisingly, we found that telmisartan treatment dose dependently increased the mitochondrial membrane potential in BV2 cells after 6

hours of treatment. The fluorescent intensity of cells incubated with TMRE increased as determined by fluorescent intensity (Figure 22A), fluorescent microscopy (Figure 22B) and flow cytometry (Figure 22C). These data show that telmisartan treatment does not negatively impact mitochondrial membrane potential, and instead, at least initially, may enhance it.

DISCUSSION

Losartan, candesartan and telmisartan have efficacy in preclinical models of traumatic brain injury (15; 272). Retrospective studies have shown that patients taking ARBs for hypertension have reduced incidence of AD and there is a growing consensus that ARBs may have efficacy in preventing or treating Alzheimer's disease (228; 273). Telmisartan is able to reduce neuroinflammation, reduce amyloid burden and improve cognitive performance in rodent AD models (152; 266). Clinical trials have begun to determine whether telmisartan is able to reduce mild cognitive impairment (290). Given the interest in these drugs to treat neurodegenerative disease and neurotrauma, an understanding on their actions in specific cell types is warranted. Here we show telmisartan has unique mechanisms in microglia to reduce inflammation. We observe that telmisartan had the most potent effect in reducing proinflammatory mediators in cultured microglia. We also showed that telmisartan is able to induce autophagy and cell death in microglia in contrast to the other ARBs, an effect that may in part account for its more potent anti-inflammatory effects but signals the need for caution.

Since ARBs work primarily by blocking AT1Rs, we assessed the possibility that AT1R mediates some of the anti-inflammatory of the ARBs. It is uncertain whether microglia express the full complement of the RAS in culture or in vivo (186). In the CNS

in vivo, it has been shown that angiotensinogen is expressed specifically in astrocytes (186; 254). In our studies, we did not observe expression of angiotensinogen mRNA in cultured microglia, although we did find low level expression of renin and ACE mRNA in the cultures (data not shown). As our microglial cultures are over 95% pure, it is probable that angiotensinogen is not expressed in the cultures. Therefore Ang II, the ligand for AT1R was not present, and we can deduce that the ARBs are not working by blocking AT1Rs. Although we detected AT1R mRNA in our cultured microglia, it is somewhat controversial whether Ang II signaling through the AT1R occurs in microglia. It was previously shown that Ang II treatment of microglia and microglial cell lines resulted in activation of iNOS, NADPH oxidase and rho kinase activity (224). However, in our hands, Ang II treatment did not induce IL1 β and TNF α mRNA in primary microglial cultures in contrast to the effects of LPS (Figure 16). We therefore conclude that the ARB-mediated anti-inflammatory effects in our microglial cultures are independent of AT1R antagonism. It is possible however that AT1R blockade plays a significant role in in vivo anti-inflammatory effects of the ARBs due to complex interactions between different cell types together with potentially different microglial phenotype in vivo than in culture (40) .

ARBs can also modulate AT1R independent signaling including through PPAR γ (20; 49; 239), PPAR α (320), PPAR δ (86) and AMPK (312). To date telmisartan is a stronger inducer of these alternate pathways than other ARBs; something our data also supports (Figures 8 and 14). In addition to telmisartan, losartan, irbesartan and candesartan act as partial PPAR γ agonists in vitro (239) and we have shown that candesartan's anti-inflammatory actions in a mouse model of TBI can be reduced with a PPAR γ antagonist (273; 274). Although PPAR γ agonists antagonize inflammation (132; 294) inhibition of

the PPAR γ pathway with T0070907, did not interfere with telmisartan's ability to reduce ROS and NO induction in LPS treated BV2 cells (Figure 18). In contrast, PPAR γ knockdown with siRNA did partially prevent telmisartan's ability to reduce TNF α and IL1 β mRNA in LPS activated microglia (312). The discrepancy between our data and those of Xu *et al* could be due to the different outcome measures used, or to the different methods of interfering with PPAR γ signaling. Taken together, we can conclude that telmisartan's ability to activate PPAR γ signaling is at most only a partial contributor to its anti-inflammatory actions.

In this study, we showed that telmisartan but not candesartan, olmesartan, valsartan and irbsartan activates AMPK in microglia. AMPK is involved in cellular responses to stress, nutrient deprivation and energy homeostasis (39) and has been associated with anti-inflammatory states in immune cells (44; 231; 312). Compound C inhibition of AMPK partially ameliorated telmisartan's ability to reduce ROS but not NO induction by LPS in microglia. Xu *et al* however showed that compound C inhibited telmisartan's ability to reduce IL1 β and TNF α mRNA in LPS activated microglia (312). We conclude that the AMPK pathway may partially account for some of telmisartan's anti-inflammatory effects in microglia.

There are several downstream pathways, which are regulated by AMPK, however no downstream or cellular effect of AMPK has been assessed in telmisartan treated microglia. mTOR is an important downstream target that is negatively regulated by AMPK (163). We showed that mTOR phosphorylation is reduced in telmisartan treated microglia (Figure 17C). Reduced phosphorylation of mTOR reduces its activity (163). mTOR mediates cell survival and proliferation with overall anabolic effects (24; 121) in contrast

to the catabolic effects of AMPK (255). The role of mTOR in regulation of inflammation is controversial (110). Inhibition of mTOR by rapamycin results in both reduction and stimulation of inflammatory mediators dependent on context (110). Treatment of dendritic cells with rapamycin resulted in activation of NF- κ B signaling and reduced IL10 production (110). On the other hand, and more consistent with our data, in microglia mTOR inhibition resulted in reduced proinflammatory cytokine release (260; 308). mTOR inhibition also results in induction of autophagy which has been shown to play significant roles in regulation of inflammation (98).

Autophagy is a cellular process involved in degradation of cytoplasmic content in order to preserve cellular homeostasis (68; 158). Although autophagy induction is principally associated with inhibition of proinflammatory processes (35; 115; 162), autophagy can be proinflammatory when it is involved in processing antigens for the adaptive immune system (58). Telmisartan has previously been shown to induce autophagy in vascular smooth muscle endothelial cells (163) and T-cells (147). We showed that telmisartan induced autophagy in BV2 microglia (Figure 19). There is an increasing body of literature indicating the anti-inflammatory effects of autophagy induction (35; 44; 114). In microglia specifically, cannabinoid receptor activation reduced neuroinflammation via activation of autophagy (244) and autophagy induction was important in A β clearance and reduction of NLRP3 inflammasome activation (53). Our data are consistent with telmisartan activating an AMPK- mTOR – autophagy pathway. Our demonstration of autophagy induction in microglia by telmisartan thus identifies a cellular process by which telmisartan may be effecting some of its anti-inflammatory and neuroprotective effects.

Telmisartan has also been shown to regulate mitochondrial function. Telmisartan increased mitochondrial activity in vascular smooth muscle cells (257), but inhibited the mitochondrial membrane polarization in T-cell leukemic cells (147). As regulation of mitochondria can increase AMPK activity through increased cellular AMP concentration, we examined whether telmisartan altered the mitochondrial membrane potential in BV2 microglia cells. The commonly used anti-diabetic drug metformin activates AMPK by increasing cytoplasmic AMP through the inhibition of the mitochondrial electron transport chain (68; 275). Our data shows that telmisartan increased the mitochondrial membrane potential (Figure 22) suggesting that telmisartan's induction of p-AMPK is not through inhibition of mitochondrial function. An alternate mechanism was suggested by Xu et al (312) who showed that telmisartan induced AMPK activation in a calcium/calmodulin-dependent protein kinase kinase β (CaMKK β) dependent manner in microglia.

A well-recognized mechanism for regulation of the immune system is the suppression of proliferation and induction of apoptosis in immune cells. Several immune modulators including TGF- β (140; 194), IL4 (317), IL13 (317) and glucocorticoids (237) inhibit cell proliferation and induce immune cell death to regulate inflammation. Telmisartan has previously been shown to induce apoptosis in a variety of cell types (64; 147; 183; 299; 313). Our data showed that telmisartan, had anti-proliferative and toxic effects on microglia (Figure 120 A,C). Telmisartan's toxicity was dose dependent with doses as low as 10 μ M causing significant toxicity as assessed by LDH release (Figure 20 B,D). More extensive cell death was observed at doses of 50 and 100 μ M. We did not detect any toxic effects of the other ARBs at 100 μ M (Figure 20A,C), showing that telmisartan's toxicity was unique. Although this reduction in microglial viability is one mechanism

through which telmisartan could have anti-inflammatory activity we do not think that this is a major anti-inflammatory mechanism for telmisartan. In the model system we employed in these studies, telmisartan's anti-inflammatory actions were on LPS stimulated microglia. Indeed it is known that LPS increase microglial cell viability (194). Telmisartan's toxicity was partially ameliorated by 3-methyladenine, an inhibitor of early autophagy (Figure 21a), and prevented by pretreatment with the pan caspase inhibitor Z-VAD-FMK (Figure 21b). These experiments suggest that telmisartan potentially mediates microglial cell death through multiple mechanisms.

Although many studies have reported that telmisartan induces death, telmisartan has a favorable safety profile (238). It is therefore possible that any cell death it may cause in clinical use is negligible, or some selective cell death effects may underlie some of telmisartan's therapeutic functions. Telmisartan has been shown to be protective of critical cells like vascular endothelial cells and neurons (94; 265). Telmisartan at 20 μ M protected neurons from glutamate mediated toxicity in vitro (279) and protects hippocampal neurons in vivo (79; 279). The reported protective effect of telmisartan in neurons (279) and its ability to reduce microglial viability is of interest to neurodegenerative diseases as telmisartan could at once be reducing toxic microglial activity by inducing apoptosis while at the same time protecting neurons.

In this study, we found that 10 μ M telmisartan increased LDH production in cultured microglia. (Figure 20 B,D). Induction of cell death by telmisartan is a potential detrimental effect. It is difficult to translate drug doses used in cell culture to those used in vivo, due to the complexity of the many factors in vivo that can influence drug

concentrations and effects. Doses and apparent potency differences may not reflect in vivo effects as metabolic processes can alter pharmacological effects. Telmisartan dose equivalent in patients is 40mg compared to 16 mg for candesartan and 50mg for losartan with similar molecular weights (2).

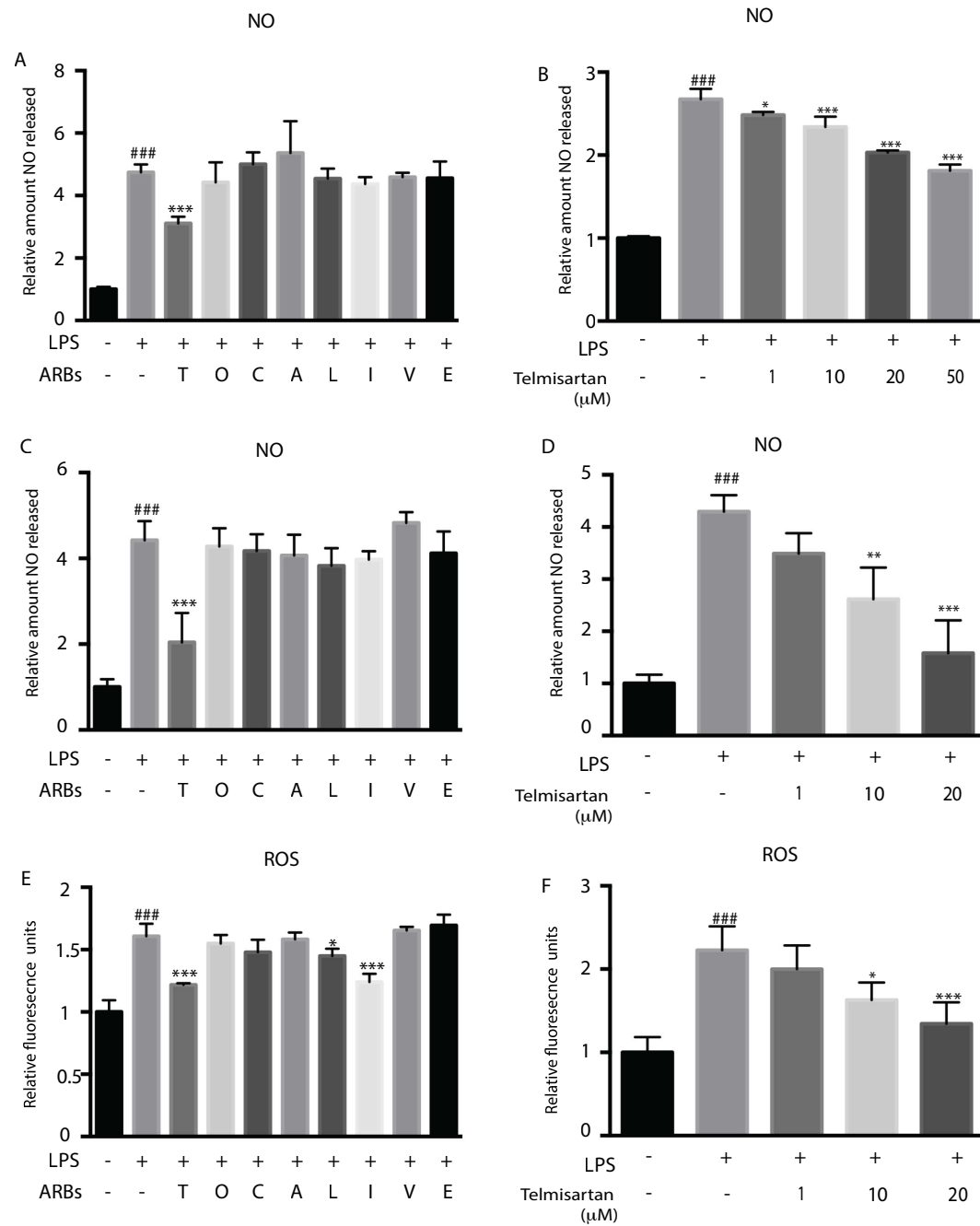
In conclusion, we have shown that telmisartan, relative to the other ARBs is a potent inhibitor of microglial activation in vitro. We showed that the PPAR γ pathway was not involved in reduction of ROS and NO in LPS activated microglia by telmisartan, however reduction in ROS by telmisartan in LPS activated microglia was partially AMPK dependent. We also show for the first time that telmisartan activates autophagy in microglia and reduces microglial viability, mechanisms which could account in part for telmisartan's anti-inflammatory effects. Future experiments would assess the role of microglial autophagy cell death and reduced proliferation due to telmisartan in in vivo neuroinflammatory states.

Table 1. Primer List.

Gene	Forward (5'-3')	Reverse (5'-3')
rIL-1 β	GACCCCAAAAGATTAAGGAT	AAAGAAGGTGCTTGGGTCCC
rT β R1	ACATCAGGGTCTGGATCAGT	CCATGAACGTTCTTCTCT
rGAPDH	ACCATCTTCCAGGAGCGAGA	GGCGGAGATGATGACCT
rSmad6	ACTGGATCTGTCCGATTCTAC	CCGGAGCTCCCAGTACGCCC
rBambi	AAGCCTCAGGACAAGGAAAC	CAAGATCAGTCCGCCAGCAT
rSnoN	CAATCAAAGACAGATACGC	GACCGCTTTAGACTGGGACC
Smad 7	AGAGTCTCCCCCTCCTCCTTA	AAGAAGTTGGGAATCTGAAG
riNOS	AAGCCCCGCTACTACTCCAT	AGCTGGAAGCCACTGACACT
miMOS	CCAAGCCCTCACCTACTTCC	CTCTGAGGGCTGACACAAGG
rTNF α	CGGTCCCAACAAGGAGGAGA	AGGAGGGCGTTGGCACGCTG

Figure 14. Telmisartan reduced anti-inflammatory mediators more effectively than other angiotensin receptor blocker.

Primary microglia (A,B) or BV2 cells (C-F) were pretreated for 2 hours with the indicated ARB followed by treatment with LPS for 12 hours (2.5ng/ml primary microglia, 100ng/ml BV2 cells). Media was collected for NO analysis by the Griess Assay (A-D) and the cells analyzed for the presence of ROS using the fluorescent indicator dye H2DCFDA (E-F). Telmisartan (T) (20 μ M) significantly reduced NO release whereas the remaining ARBS (100 μ M) olmesartan (O), candesartan (C), Azilsartan (A), Losartan (O), Irbesartan (I), Valsartan (V) and Eprosartan (E) had minimal effects (A,C). Telmisartan dose dependently reduced NO in primary microglia (B) or BV2 cells (D). Telmisartan, Losartan and Irbesartan significantly reduced ROS generation in BV2 microglia (E). Telmisartan dose dependently reduced ROS in BV2 cells (F). (Data are representative of at least 3 repeated experiments, n=4 per experiment. ***p < 0.001, **p < 0.01, *p < 0.05, compared with LPS treated cells; ### p < 0.001, compared with vehicle).induction of IL1 β mRNA (B) (n=4, ***p<0.001).



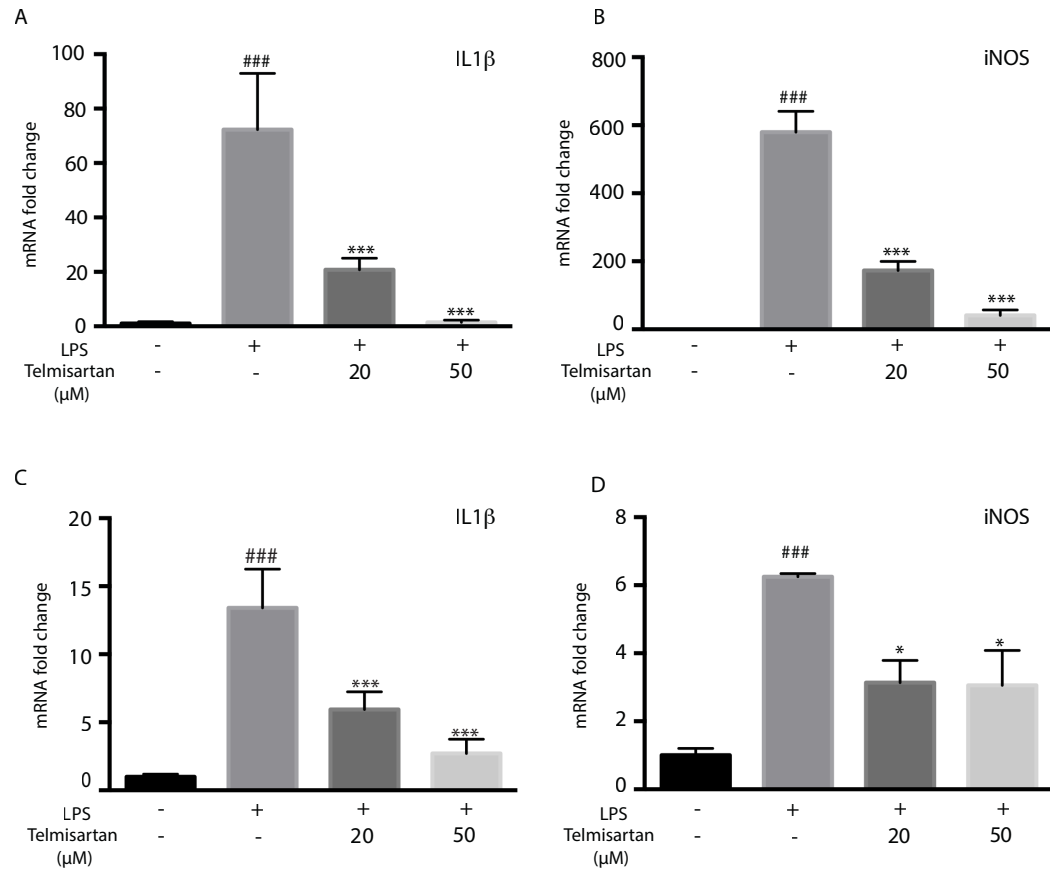


Figure 15. Telmisartan reduces mRNA of proinflammatory mediators.

Primary microglia (A,B) and BV2 cells (C,D) were pretreated with telmisartan for 2 hours followed by LPS (2.5ng/ml primary microglia and 100ng/ml for BV2 cells) for 12 hours. The cells were then harvested and RNA isolated for qPCR. Telmisartan dose dependently reduced iNOS (B,D) and IL1 β (A,C) BV2 and primary microglia. (Data are representative of at least 3 repeated experiments, n=4. ***p < 0.001, **p < 0.01, *p < 0.05, compared with LPS treated cells; ### p < 0.001, compared with vehicle).

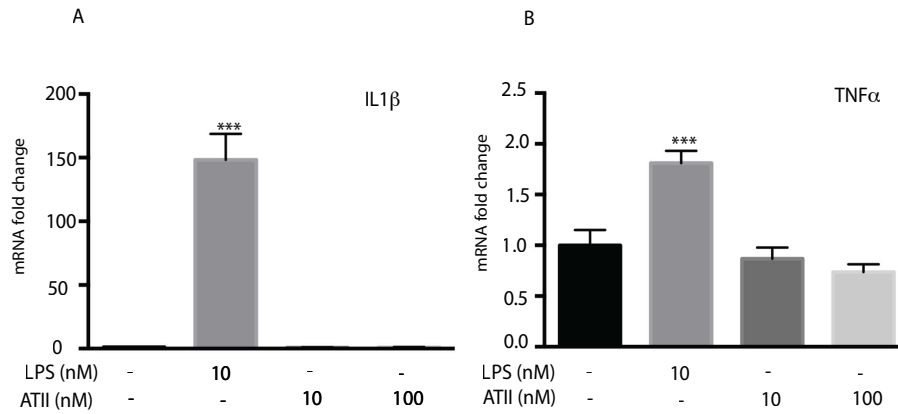


Figure 16. Angiotensin II did not directly induce a proinflammatory response of microglia in culture.

Primary microglia were treated with either Angiotensin II (ATII) or LPS for 12 hours before harvesting and isolation of RNA. qPCR indicated that ATII did not detectably alter IL1 β or TNF α expression. (Data are representative of at least 2 repeat experiments; n=4, mean $\pm$ s.e.m. ***p<0.001).

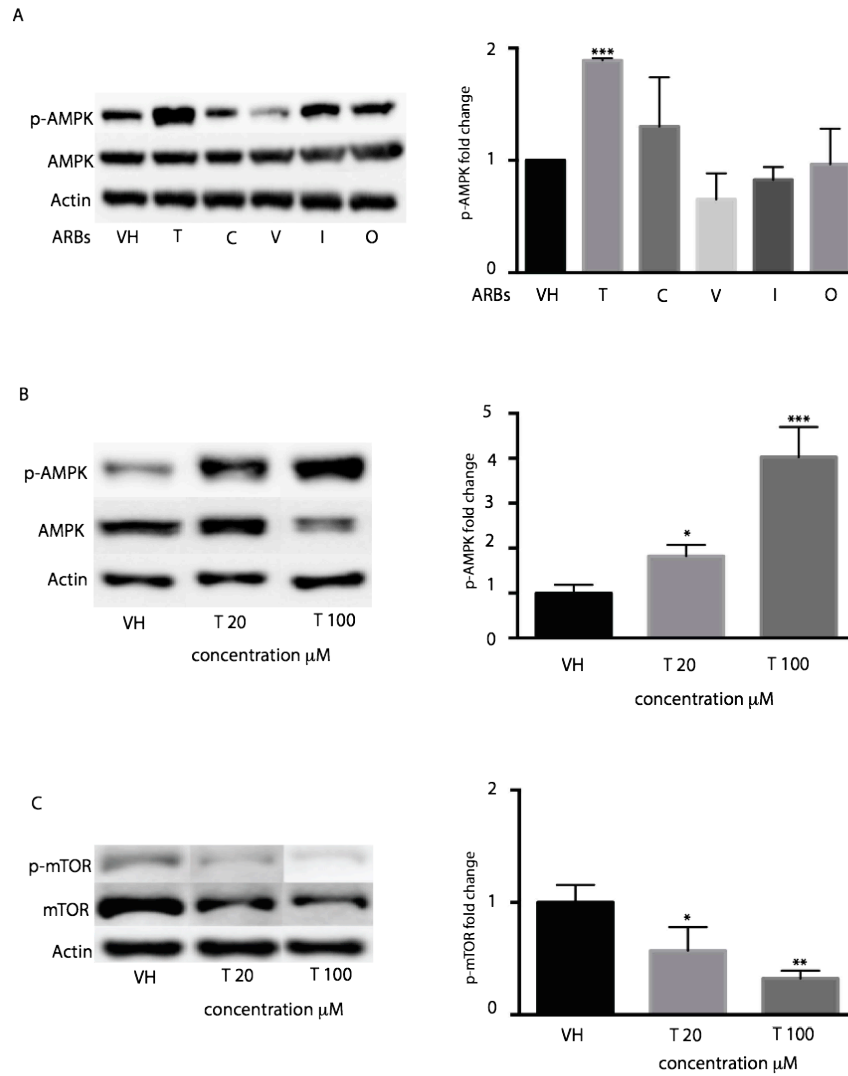


Figure 17. Telmisartan activates the AMPK/mTOR pathway in BV2 microglia.

BV2 microglia were treated with the indicated ARBs (10 μ M) for 3 hours before harvest and isolation of proteins. Western blots indicate the effect of treatment on AMPK and mTOR phosphorylation. Telmisartan (T) but not candesartan (C) olmesartan (O), valsartan (V), or Irbesartan (I) significantly induced phosphorylation of AMPK (A). Telmisartan treatment resulted in dose dependent phosphorylation of AMPK (b) and reduction in mTOR phosphorylation (c). (Data are representative of at least 2 repeat experiments. n=3, mean $\pm$ s.e.m. **p<0.01, *p<0.05).

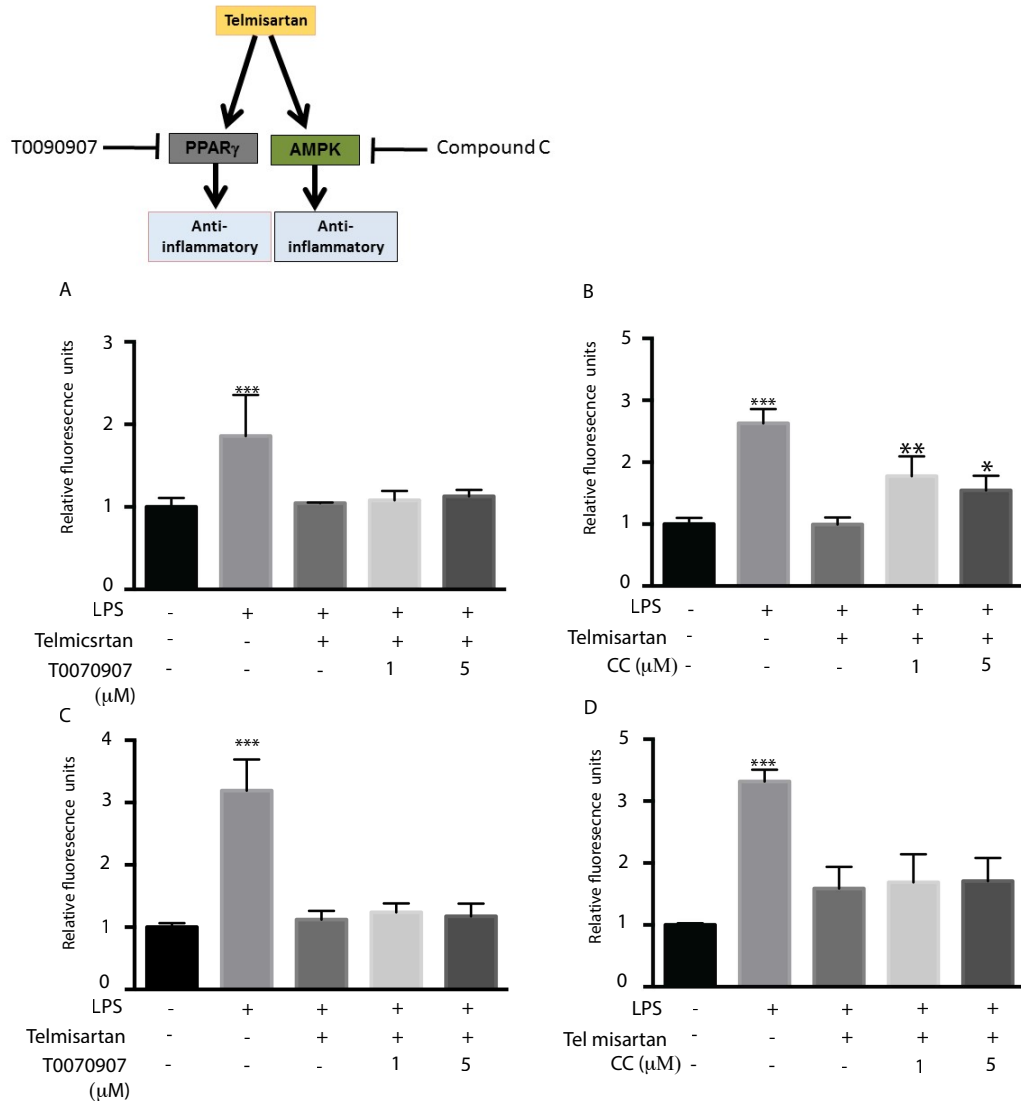


Figure 18. Inhibition of AMPK by compound C reduced ROS but not NO release from microglia.

BV2 microglia were pretreated with AMPK inhibitor compound C (CC) (1 μ M and 5 μ M) or PPAR γ inhibitor T0070907 (1 and 5 μ M) for 1 hour followed by Telmisartan (20 μ M) for 2 hours (Schematic above graphs). Cells were then treated with LPS (100ng/ml) for 12 hours. Medium was collected for analysis of NO content by Greiss Assay and the cells analyzed for ROS using H2DCFDA. Compound C (B) but not T0070907 (A) prevented the telmisartan dependent reduction of ROS in LPS treated BV2 microglial cells. However, neither Compound C nor T0070907 altered the telmisartan-mediated reduction in LPS induced NO release (C,D). (Data are representative of at least 3 repeat experiments, n=4 mean $\pm$ s.e.m. ***p<0.001, *p<0.05).

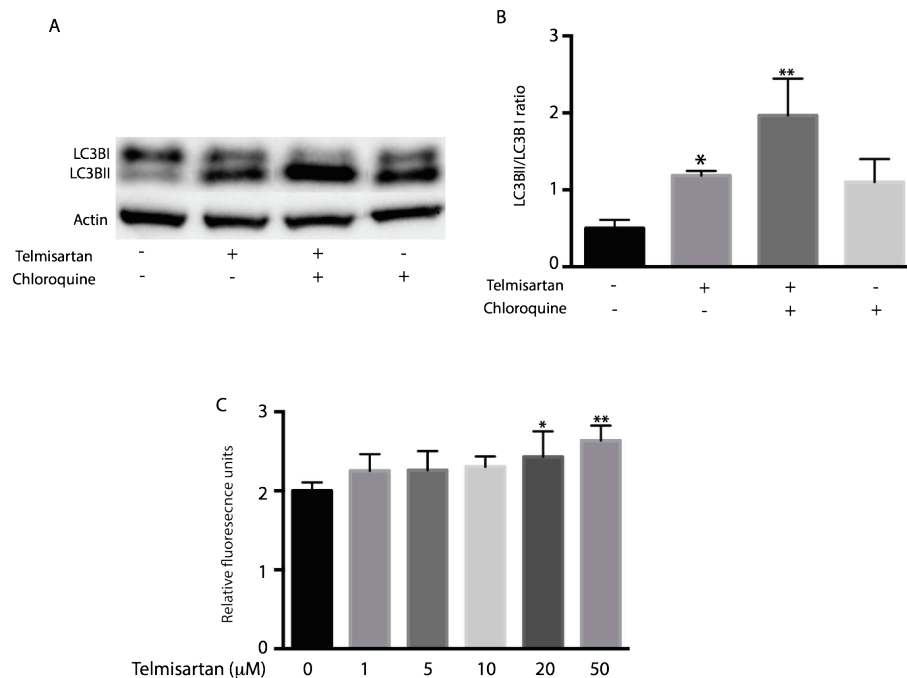


Figure 19. Telmisartan induces markers of autophagy in BV2 microglia.

BV2 cells were treated for 10 hours with telmisartan (20μ M) with or without chloroquine (50μ M) before harvesting and protein extraction. Western blot of LC3B shows that telmisartan increased the ratio of LC3BII to LC3BI. This increase was augmented by chloroquine treatment (A). Autophagy was also assessed using the CytoID kit. Telmisartan dose dependently increased fluorescence of BV2 cells incubated with the CytoID dye, indicating increased autophagosome formation. (Data are representative of 3 repeated experiments (Mean $\pm$ s.e.m, $n=3$, *** $p < 0.001$, * $p < 0.05$)).

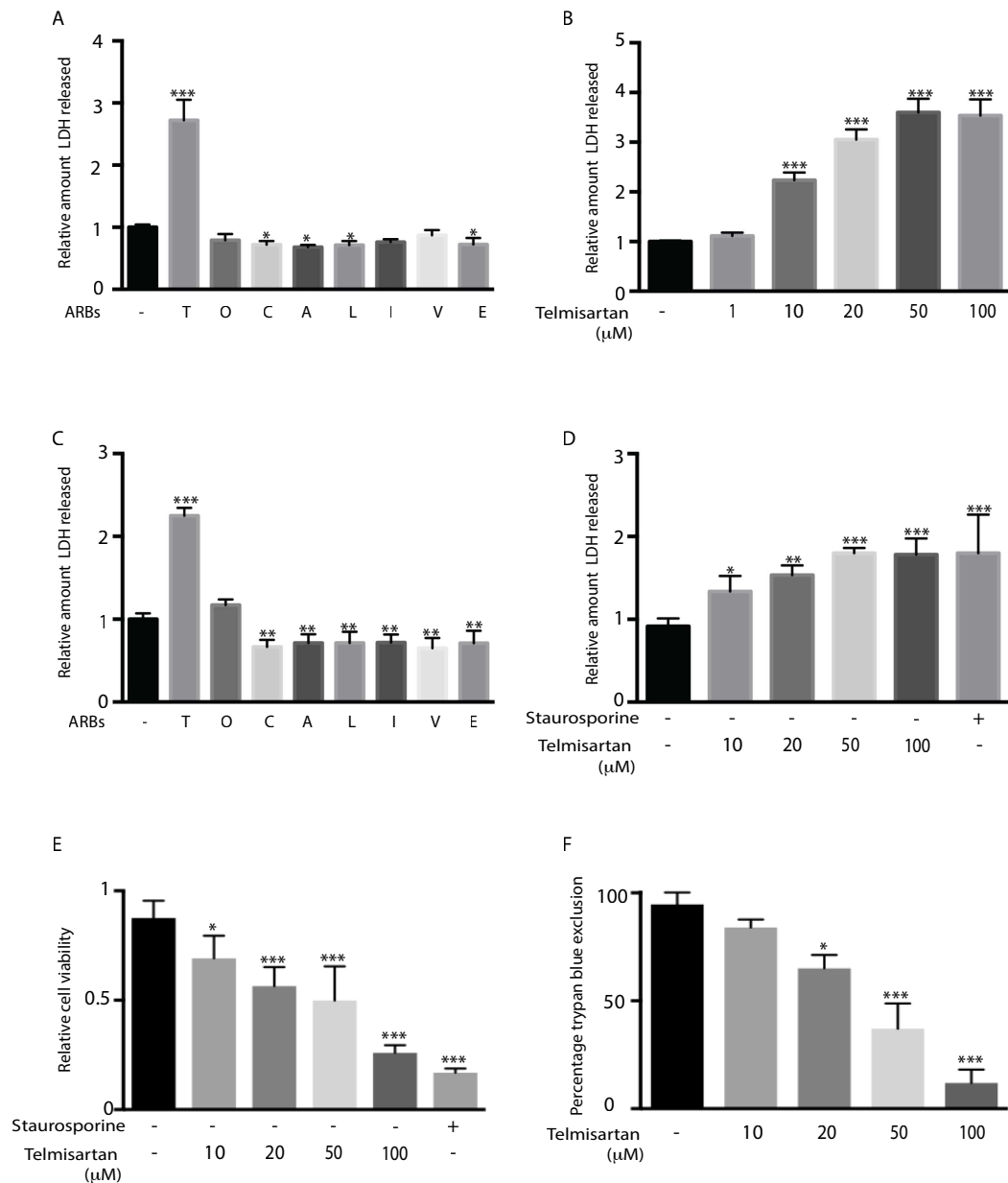


Figure 20. Telmisartan induces death of BV2 and primary microglia.

Primary microglia and BV2 cells were treated with different ARBs (100 μ M) for 12 hours before assessment of LDH release and cell viability. Telmisartan was the only ARB to significantly induce LDH release in either primary microglia (A) or BV2 cells (C). Telmisartan dose dependently increased LDH release in primary microglia (B) and BV2 cells (D). Staurosporine (1 μ M) was added as a positive control for cell death (D,E). Telmisartan also dose dependently reduced cell viability and proliferation indicated by the amount of reduced MTS detected (E) and by trypan blue exclusion (F). (Data are representative of at least 3 repeat experiments, n=4 mean $\pm$ s.e.m. ***p < 0001, **p<001, *p<05).

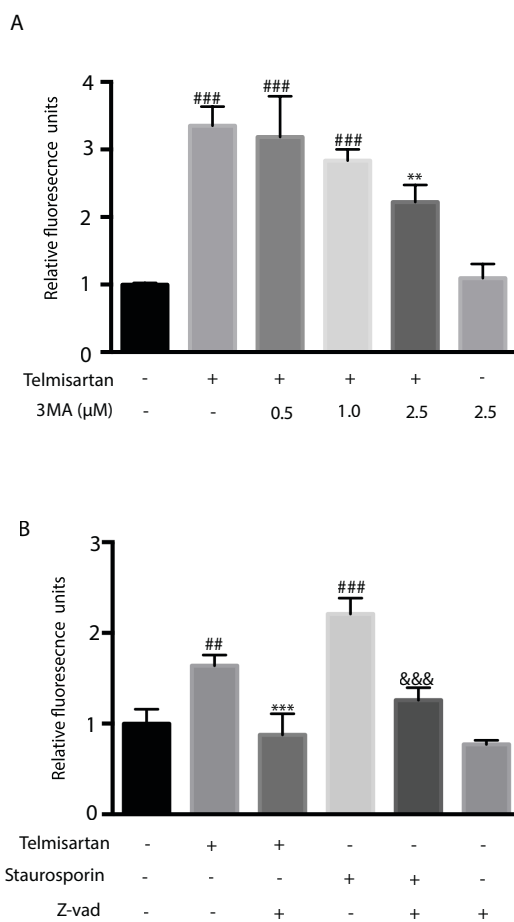


Figure 21. Inhibition of autophagy and the apoptotic pathway reduced telmisartan's death inducing effects.

A. BV2 cells were pretreated with 3MA (0.5, 2 and 2.5 μ M) for 1 hour followed by telmisartan 50 μ M for 12 hours. Media were then collected for LDH assay. LDH was significantly increased with telmisartan treatment (### $p < 0.001$). 3MA at 2.5 μ M concentration partially reduced telmisartan mediated LDH release (** $p < 0.001$). B. BV2 cells were pretreated with Z-VAD-FMK (20 μ M) for 1 hour followed by 50 μ M of telmisartan or 0.5 μ M Staurosporine for 12 hours. Telmisartan and Staurosporine significantly induced LDH release (#### $p < 0.001$, ## $p < 0.001$). Z-VAD-FMK also significantly reduced telmisartan (*** $p < 0.001$) and staurosporine (&&& $p < 0.001$) mediated increase in LDH. (Data are representative of 2 repeated experiments (Mean $\pm$ s.e.m, n=3)).

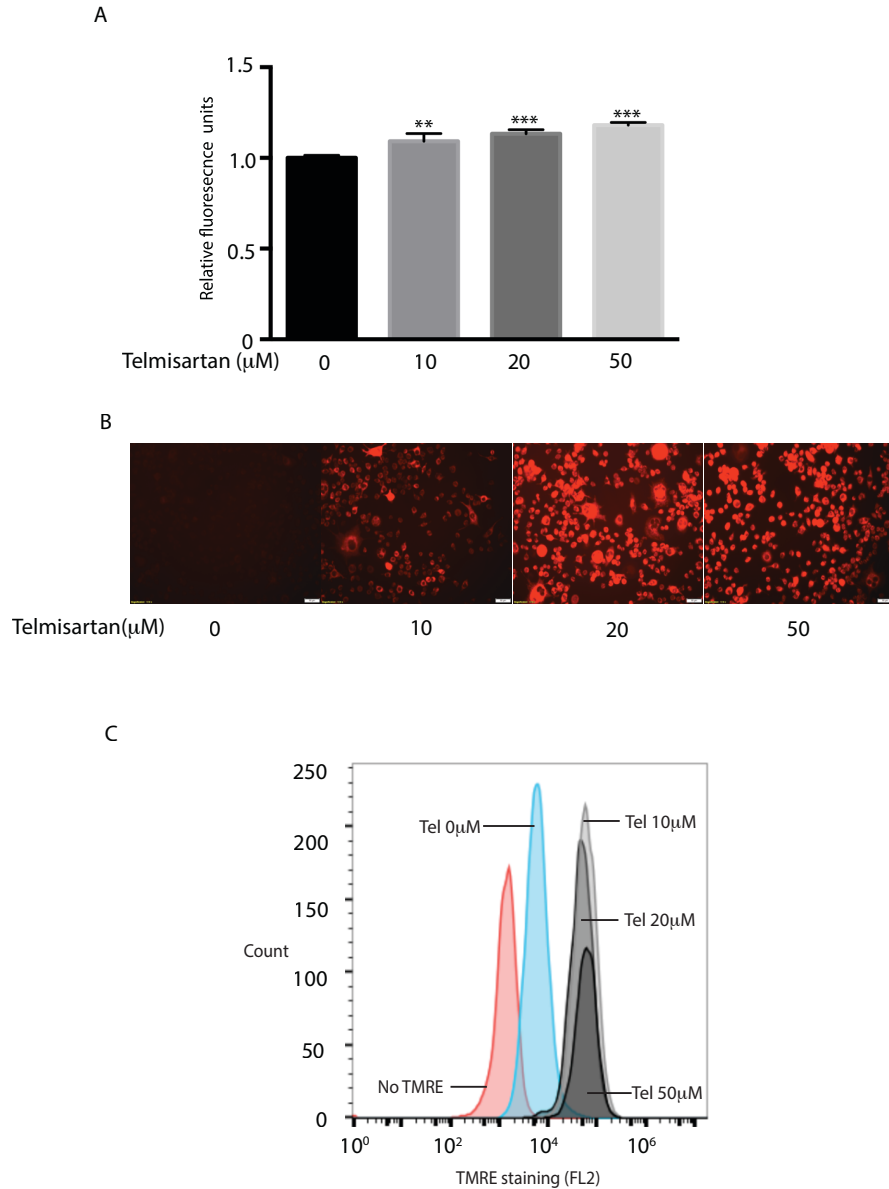


Figure 22. Telmisartan increases microglial membrane potential in BV2 cells.

(A). BV2 microglia were treated with increasing doses of telmisartan (Tel) in 96 well plates for 6 hours. The cells were then treated with TMRE (1μM) for 20 minutes followed by fluorescence emission reading in a plate reader. Telmisartan dose dependently increased TMRE fluorescence emission (***P<0.001, **P<0.01). (B) Representative florescent images of telmisartan treated BV2 cells were taken with a florescent microscope after reading in plate reader. (C) BV2 cells were plated in 6 well plates and treated with increasing doses of telmisartan for 6 hours followed by 20 minutes of TMRE treatment. The cells were harvested and prepared for flow cytometry. All the telmisartan doses increased the intensity of TMRE in treated cells. (Data are representative of 2 repeated experiments).

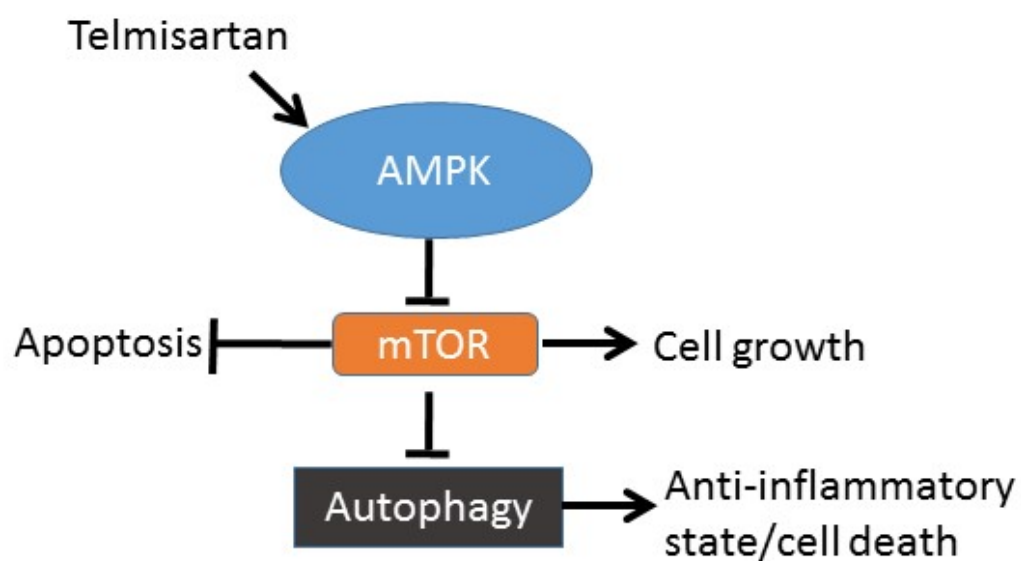


Figure 23. Schematic of telmisartan's effect on AMPK, mTOR and autophagy.

Activation of AMPK by telmisartan leads to reduction in mTOR phosphorylation. Reduction in mTOR activation by telmisartan was associated with activation of autophagy and reduction in proinflammatory mediators and cell death.

CHAPTER 4: Discussion

The prevalence of neurodegenerative disorders, of which AD is most common, is increasing worldwide with no effective therapeutic options despite extensive research (1; 8; 278). Chronic neuroinflammation mediated mainly by persistent microglial activation has been strongly associated with neurodegeneration (10; 303). In this thesis, I have studied two separate mechanisms, TGF- β pathway and the RAS systems, by which chronic microglial activation can be tackled for therapeutic gains in chronic neuroinflammation and neurodegeneration. I focused on improving understanding of how the anti-inflammatory TGF- β signaling is inhibited in activated microglia and how RAS modulation with ARBs can be beneficial in ameliorating microglial activation.

Our previous study showed that the anti-inflammatory TGF- β signaling pathway is inhibited after treatment of microglia with LPS in vitro (194) leading us hypothesize that microglia may be chronically activated in AD and other neurodegenerative disorders in part, because of inhibition of anti-inflammatory signaling pathways. In this study, we went further to show that antagonism of TGF- β signaling in activated microglia does not only occur with LPS treatment but also with A β , the pathogenic protein in AD, and HKLM. We showed that treatment of microglia with A β resulted in increased survival and a reduction of TGF- β mediated cell death. We also began to assess the molecular pathways by which proinflammatory mediators inhibit TGF- β signaling in activated microglia and showed that the NF- κ B plays a critical role.

The second part of this thesis assesses the anti-inflammatory effects of FDA approved ARBs on activated microglia. We observed that telmisartan was the most potent of the ARBs in reducing proinflammatory mediator release from microglia in vitro. We

also showed that telmisartan reduced AMPK phosphorylation, reduced mTOR activation and induced autophagy in microglia. Finally we observed that of the ARBs, telmisartan reduced microglial viability, which is another potential mechanism for its anti-inflammatory and neuroprotective effects.

TβR1 mRNA IS REDUCED IN ACTIVATED RAT MICROGLIA

TβR1 is a key receptor in downstream canonical TGF-β signaling from the cell membrane (182). TβR1 is also a target for down regulation in LPS activated primary microglia (194). TβR1 is critically regulated in TGF-β signaling by several proteins including Smad7, Smurf1 and FKBP2 (50; 182). As it is known that there is increased Aβ and defective TGF-β signaling in neurons in AD (262), we sought to determine whether antagonism of TGF-β signaling also occurred in microglia exposed to Aβ. We replaced LPS with Aβ to determine whether Aβ could down regulate TβR1 in primary microglia in a similar manner as LPS. Aβ treatment did lower TβR1 mRNA in primary microglia just like LPS, indicating that negative regulation of TGF-β signaling may be a common effect in activated microglia. As both LPS and Aβ activate TLR4 it is not surprising that they also both decrease TβR1 (192; 258). Aβ however also activates scavenger receptors distinct from TLR4 (81). We however did not assess the added effect of non TLR4 signaling in regulation of TβR1 in Aβ activated microglia. We also treated microglial with HKLM, a TLR2 receptor agonist (245) and observed a similar down regulation of TβR1 mRNA, suggesting further that TβR1 down regulation is not restricted to TLR4 activation. We speculate that many of the other proinflammatory pathways when activated in microglia, could lead to a reduction in TβR1 and TGF-β signaling. This could, therefore, have implications for many neurodegenerative diseases associated with microglial activation

like AD, Parkinson's disease and neurodegeneration associated with CNS trauma and infections.

ACTIVATION OF MICROGLIA RESULTS IN REDUCTION IN TGF- β MEDIATED GENE INDUCTION

TGF- β regulates the expression of many genes depending on context. In this thesis, I assessed regulation of two TGF- β induced genes, T β R1 and Smad6, in activated primary microglial to determine how their expression is altered. Although T β R1 serves as receptor for TGF- β , T β R1 is also induced by TGF- β and thus serves to augment signaling through the pathway (194). In our experiments, we observed that besides decreasing T β R1 mRNA and protein in microglia, A β also prevented upregulation of TGF- β mediated induction of T β R1 mRNA and protein.

Although the inhibition of TGF- β signaling was observed in vitro, it is possible that a similar situation could prevail in vivo in neurodegenerative disorders. It has been observed in sufferers of neurodegenerative disorders like AD (43; 259; 262) and Parkinson's disease (271) that the TGF- β levels in the CSF is increased although there is persistent microglial activation. This observation suggests that there could be inhibition of TGF- β signaling in microglia in AD like has been observed for neurons (62; 262).

Inhibition of T β R1 induction by A β in microglia implies that not only is there a reduced ability of TGF- β to protect neurons in AD (262), there may also be a secondary neurotoxic effect due to inability of TGF- β to reduce microglial activation as well and thus resulting in release of neurotoxic inflammatory mediators. In vivo studies are needed to access the extent to which microglial TGF- β signaling is impaired in neurodegenerative disorders, as it could aid in discovery of potential therapeutic targets.

TGF- β mediated induction of Smad6 mRNA, like T β R1, was also reduced in A β and LPS activated microglia. Although Smad6 is involved in negative regulation of signaling by members of the TGF- β super family, its regulatory effects are more associated with BMP2 signaling (116). Smad6 is also a mediator of the anti-inflammatory effects of TGF- β . TGF- β induced Smad6 inhibits TLR4 signaling by recruiting E3 ligase Smurf3, targeting Myd88 for degradation (160). Smad6 can also bind to Pellino-1 and inhibit IRAK signaling which interrupts MyD88 dependent TLR4 pathway (54). Prevention of TGF- β mediated induction of Smad6 by A β and LPS in activated microglia thus represents a direct inhibition of an anti-inflammatory mechanism of TGF- β and strengthening the point that anti-inflammatory responses are compromised in chronic neuroinflammation.

Although TGF- β signaling in microglia is generally beneficial, on the whole, persistently elevated TGF- β in the CNS has deleterious effect, as sustained elevation of TGF- β in the CNS can induce amyloid angiopathy (113; 301). Thus, strategies aimed at augmenting TGF- β signaling pathways in specific cells may be a reasonable therapeutic goal rather than further increasing TGF- β in the brains of sufferers of neurodegenerative diseases. Augmentation of a TGF- β induced anti-inflammatory effect to mitigate inflammation was demonstrated by Lee *et al* when they developed a Smad6 variant protein Smaducin6 to reduce lethal systemic inflammation (159). Such a strategy can be adopted in the CNS in an effort to increase a specific anti-inflammatory branch of TGF- β signaling without the need to further increase total levels of TGF- β in the brain.

AB OVERRIDES TGF- β MEDIATED REDUCTION IN MICROGLIAL CELL VIABILITY

In the injured brain and neurodegenerative disorders, there is increased microglial proliferation with an associated increased population of activated microglia (28; 101). The anti-inflammatory machinery in CNS injury and neurodegeneration is mediated by anti-inflammatory cytokines including IL4, IL13 and TGF- β , which not only attempt to reduce pro-inflammatory cytokine release from activated microglia but also can reduce microglial population by inducing microglial cell death (194; 256; 317). The fact that the population of activated microglia is increased in neurodegenerative disease and aging, in spite of the presence of inhibitors of microglial proliferation like TGF- β , may suggest some resistance to TGF- β mediated anti-proliferative and pro-apoptotic effects. In my first paper, we showed that TGF- β treatment, as expected, lead to death of primary microglia over time. We however observed an increased population of primary microglia after A β treatment and this increase was not reduced when primary microglia were treated with both A β and TGF- β . This suggests that A β treated microglia are resistant to TGF- β mediated cell death.

Although TGF- β signaling in microglia is generally beneficial, on the whole, persistently elevated TGF- β in the CNS has deleterious effect, as sustained elevation of TGF- β in the CNS can induce amyloid angiopathy (113; 301). Thus, strategies at augmenting TGF- β signaling pathways in specific cells may be a reasonable therapeutic goal rather than further increasing TGF- β in the brains of sufferers of neurodegenerative diseases. Augmentation of a TGF- β induced anti-inflammatory effect to mitigate inflammation was demonstrated by Lee *et al* when they developed a Smad6 variant protein Smaducin6 to reduce lethal systemic inflammation (159). Such a strategy can be adopted in the CNS in an effort to increase a specific anti-inflammatory branch of TGF- β signaling without the need to further increase total levels of TGF- β in the brain.

Activation of the NF- κ B pathway maintains the survival of macrophages and microglia (119; 211). The increased survival of microglia by A β may therefore be a direct effect of increased NF- κ B signaling and reduced Smad6 expression as we observed (Figure 8B), as normal Smad6 levels would otherwise have antagonized the NF- κ B pathway and reduced survival. This suggests that the use of a protein like smaducin6 could be of benefit in chronic neuroinflammation not only by reducing proinflammatory cytokine release but also inhibiting a pro-survival NF- κ B pathway and thus may augment the suppressive effect of circulating CSF TGF- β on microgliosis. An alternative approach at limiting the population of activated microglia in neurodegeneration would be the use of drugs that are known to inhibit microglial proliferation and reduce their survival. Such a scenario is addressed in my second paper where I looked at potential anti-proliferative effects of ARBs.

INHIBITION OF NF- κ B PARTIALLY PREVENTS DOWN REGULATION OF T β R1 IN ACTIVATED MICROGLIA

In order that new therapeutic strategies can be developed to ameliorate chronic neuroinflammation based on modulation of altered TGF- β signaling, it would be necessary to understand the molecular mechanisms that underlie crosstalk between proinflammatory pathways like TLR4 and TGF- β signaling in activated microglia. It has been shown in fibroblasts that LPS can inhibit TGF- β signaling via NF- κ B activation (27). In T-cells, activation of the T cell receptor resulted in inhibition of TGF- β signaling and Smad phosphorylation, via the protein kinase C θ pathway (96). Kim *et al* showed that in RAW macrophage cell lines, LPS inhibited TGF- β signaling via activation of ERK (141) while Xu *et al* showed that the IFN γ pathway could inhibit TGF- β signaling in T lymphocytes (310). In this study we showed that inhibition of the NF- κ B pathway but not the ERK or

IFN3 prevented LPS and A β mediated down regulation of T β R1 mRNA in activated microglia.

Because inhibition of a pathway involves more than simple changes in mRNA, it is possible that TLR4 mediated antagonism of TGF- β signaling could occur via mechanisms which were not explored in this study. For example TLR4 signaling could inhibit TGF- β signaling in microglia via alterations in protein interactions or phosphorylation. Further studies will have to examine more direct signaling changes including alterations in protein interactions.

In this thesis I also assessed induction of mRNA of some TGF- β antagonistic genes Bambi, SnoN and Smad7 and observed that Bambi and SnoN were transiently induced in A β and LPS treated microglia. Interestingly we observed no induction in Smad7 mRNA. Smad7 has however been shown to be involved in inhibition of TGF- β signaling in an NF- κ B dependent manner (27). Further studies here as well will require analyzing protein changes and interactions for a clearer understanding of the mechanism of endogenous TGF- β pathway inhibitors in inhibiting TGF- β signaling in neuroinflammation.

TELMISARTAN MORE POTENTLY REDUCES PROINFLAMMATORY MEDIATORS IN ACTIVATED MICROGLIA

Recent strategies being adopted for the treatment or amelioration of brain injury and neurodegeneration is the repurposing of FDA approved drugs that show potential neuroprotective or anti-inflammatory effects. In my second study, we assessed the available FDA approved ARBs for their ability to reduce microglial activation. In vitro, telmisartan at 20 μ M more potently reduced proinflammatory mediators than the other ARBs at 100 μ M. Telmisartan also dose dependently reduced the induction of iNOS and IL1 β by LPS as has been previously described (267; 312). Given that NO generation and

the formation of reactive nitrogen species is quite detrimental to neurons (37), telmisartan may be better at protecting neurons in vivo from reactive nitrogen and oxygen species than the other ARBs. It must however be considered that in vitro effects do not always reflect in vivo states as metabolism and other in vivo processes could affect an ARBs anti-inflammatory property. For example, Losartan is metabolized in the liver to EXP3174 which has more than 10-fold the AT1R blocking property of un-metabolized losartan and EXP3174 could contribute in vivo to inhibit inflammation. Another product of Losartan metabolism, EXP3179 has no AT1R antagonist activity but has strong anti-inflammatory properties including NF- κ B, COX2 and AP-1 inhibition (133; 134; 225).

ANTI-INFLAMMATORY EFFECT OF THE ARBs IN MICROGLIA IS INDEPENDENT OF AT1RS

Given that all the ARBs are potent antagonists of AT1R (134), they could all potentially reduce microglial activation if activation is AT1R dependent. Although some ARBs have been shown to have anti-inflammatory effects in vivo in the CNS (272), it is not clear whether the anti-inflammatory effects of the ARBs are due to direct effects of blocking AT1Rs on immune cells like microglia. The ARBs could also mediate their anti-inflammatory effects through non-AT1R pathway in microglia. Ang II treatment of primary microglia did not lead to upregulation of TNF α and IL1 β mRNA compared to LPS, suggesting that Ang II does not directly activate microglial in culture. ARBs may therefore be working through AT1R independent pathways.

TELMISARTAN ACTIVATES AMPK AND REDUCES mTOR ACTIVATION

Besides their ability to block AT1Rs, some of the ARBs have been shown to activate other anti-inflammatory pathways. Irbesartan, telmisartan, losartan and candesartan have partial PPAR γ agonist activity (76). Telmisartan also activates the AMPK pathway

(243; 312) and reduces the activation of mTOR downstream of AMPK in vascular smooth endothelial cells (163). AMPK activation has anti-inflammatory effects (229; 231). In this study, we showed that treatment of BV2 cells with telmisartan resulted in increased phosphorylation of AMPK as has previously been shown in microglial cells (312). We also tested other ARBs including candesartan, valsartan, irbesartan and olmesartan for their ability to activate AMPK however none of them showed any AMPK activation at the dose used.

The AMPK pathway is a master regulator of metabolism and it is activated in circumstances of cellular stress and reduction in cellular energy production characterized by increased AMP/ATP ratio (98). Increased AMP in the cell triggers activation of AMPK (39) through the activation of the protein LKB1(275). An alternative mechanism of AMPK activation is through phosphorylation by CaMKK β (275; 312). Several drugs with pro-metabolic and anti-inflammatory effects like metformin, the first line drug for treatment of type 2 diabetes (275), and thiazolidinediones (157), also used in diabetes, activate AMPK (166; 253). Metformin activates AMPK by inhibiting the mitochondrial enzyme complex I and hence increasing the cellular AMP levels (275). The effect of telmisartan on mitochondrial activity appears to vary depending on context. Telmisartan enhanced mitochondrial activity in coronary endothelial cells (153). Telmisartan also enhanced mitochondrial activity by activating mitochondrial complex II and increasing ATP production in vascular smooth muscle cells (257). However a recent study showed that in T-cells leukemia cells, telmisartan reduced mitochondrial membrane potential and function indicated by reduced mitochondrial membrane polarization with telmisartan treatment (147). The apparent contrasting effects of telmisartan on mitochondrial may be due to

duration of treatment as higher doses used over a long periods may initially increase mitochondrial activity but may ultimately reduce once cells decline in viability. Telmisartan has previously been shown to activate AMPK via CaMKK β in microglia (312) however, the possibility that it could also activate AMPK via the mitochondria led us to assess its effect on mitochondrial membrane polarization. Interestingly, our data showed that in BV2 microglia, telmisartan treatment resulted in increased mitochondrial membrane potential and thus suggesting that telmisartan's ability to activate AMPK is unlikely to be via mitochondrial inhibition and increased AMP, in contrast to metformin. A further more detailed analysis of telmisartan on mitochondrial function in microglia is warranted, including looking at biogenesis and enzyme activity, as mitochondrial function affects many cellular processes including cell death, an effect telmisartan has been shown to regulate.

Although AMPK activation in microglia by telmisartan reduced inflammation and M2 phenotype polarization (312), the downstream pathways and cellular processes involved have however not been assessed. In this study, we showed that activation of the downstream target of AMPK mTOR, as expected, was reduced in BV2 cells treated with telmisartan. We however did not determine whether the reduction in mTOR activation was a direct effect of AMPK activation in this study as an AMPK independent pathway is also possible.

The mTOR pathway, like the AMPK pathway, is highly involved in cellular metabolism with multiple and complex interactions with several cellular processes (24). In metabolism, mTOR functions are antagonistic to AMPK. AMPK is activated during cellular nutrient deprivation and is essentially catabolic (255); mTOR however is activated

in the presence of increased nutrients and couples that with increased cell proliferation and survival and is therefore generally anabolic (287). The effect of mTOR activation on inflammation is however not clear cut as it appears to be context dependent (285; 287). While it has been shown that mTOR activation reduced TLR mediated inflammatory responses (32), mTOR inhibition could lead to activation of some proinflammatory processes as well (285). Weichart *et al* (2011) showed that inhibition of mTOR can reduce the anti-inflammatory effects of glucocorticoids (286) and inhibition of mTOR can increase the release of proinflammatory mediators from innate immune cells and macrophages (287). Inhibition of mTOR using rapamycin however reduced microglial activation in vitro and in vivo (114; 260; 308). Telmisartan's ability to reduce mTOR in BV2 microglia may therefore indicate a potential anti-inflammatory mechanism although further studies would be necessary to elucidate this.

In this study, we assessed whether inhibition of AMPK using compound C would inhibit telmisartan's ability to reduce LPS induced NO or ROS expression. We observed a partial inhibition of the ability of telmisartan to reduce ROS but not NO, in BV2 cells. Compound C has previously been shown to inhibit telmisartan's ability to reduce IL1 β and TNF α mRNA in LPS activated BV2 cells (312). We however did not observe any effect of inhibition of PPAR γ on telmisartan's ability to reduce LPS mediated ROS and NO release although PPAR γ is one of the known anti-inflammatory pathways activated by telmisartan (20). The molecular mechanism of telmisartan in reducing LPS mediated ROS and NO release may therefore be PPAR γ independent and partially AMPK dependent.

TELMISARTAN INDUCES AUTOPHAGY IN BV2 MICROGLIA

Autophagy is a key cellular process involved in lysosomal mediated degradation of cellular content leading to the recycling of macromolecules to ensure cellular homeostasis (17). Autophagy is linked to inflammation in complex ways, which are not yet completely understood (68; 322). The anti-inflammatory role of autophagy centers mainly on inhibition of the function of inflammasomes (115; 327). Autophagy can play an enabling role in inflammation when it participates in antigen processing and presentation in macrophages to activate adaptive immunity (236). Autophagy is however considered to be overall, more anti-inflammatory in its effects as disruption of autophagy leads to increased inflammation and is associated with autoimmunity (68; 322). In microglia, induction of autophagy is associated with reduction in inflammatory mediator release (35; 114). One of the common pathways for activation of autophagy is by activation of AMPK which inhibits mTOR (98). mTOR is a potent inhibitor of autophagy and therefore mTOR inhibition leads to autophagy activation (24). Autophagy was assessed by LC3BII to LC3BI ratio by western blot and cytoID, which measures the presence of autophagic vacuoles in the cytoplasm using fluorescence. Our results showed that the telmisartan mediated AMPK activation and reduction in mTOR activation was associated with an increase in induction of autophagy. Although we were not able to experimentally establish the mechanistic link between AMPK, mTOR and autophagy, we surmise that the pathway could be involved as was shown in telmisartan treated vascular smooth muscle cells (163). It is also however possible that other pathways to autophagy besides AMPK could be involved.

Autophagy shares many processes and proteins with the phagocytic system (123; 144; 162). LC3 for example is a critical protein for autophagy but is also key to LC3 mediated phagocytosis also referred to as non-canonical autophagy (180; 187). Indeed, in

the phagocytic pathway, phagosomes are sometimes fused into autophagosome before being fused into a lysosome to form an autophagolysosome in contrast to phagolysosomes which are formed by direct fusion of phagosomes and lysosomes in canonical phagocytosis, or autolysosomes which are a fusion of autophagosomes and lysosomes (144). Induction of autophagy can therefore augment phagocytic processes.

In traumatic brain injury, AD, and other neurodegenerative disorders, there is a defect in clearance of abnormal proteins from the CNS parenchyma (41). The impaired clearance of misfolded proteins is associated with impaired autophagy in neurodegenerative disorders (188; 213). Augmentation of autophagy improves clearance of A β plaques by cultured microglia and impaired autophagy caused impairment in A β clearance in an AD mouse model (53). In a mouse model of AD, telmisartan administered intranasally was shown to reduce neuroinflammation, intracellular A β and parenchymal senile plaques (266). Although it has not been assessed, telmisartan could be augmenting A β clearance by augmenting autophagy. Induction of microglial autophagy by telmisartan could therefore be a reasonable therapeutic target in AD.

Besides reducing neuroinflammation, telmisartan also has direct neuroprotective effects in vitro (279) as well as in vivo (266). Autophagy in neurons is very important for neuronal health (158). Although it has not been assessed whether telmisartan can induce autophagy in neurons, it is however possible that the observed neuroprotective effects of telmisartan may in part be due to its ability to induce neuronal autophagy and this hypothesis would require testing in future studies.

TELMISARTAN INDUCES CELL DEATH OF MICROGLIA

One of the mechanisms by which the immune system is modulated is by the induction of cell death of immune cells (85; 176; 324). There are several mechanisms of programmed cell death induction including apoptosis (75), necroptosis (288) and pyroptosis (88). Apoptosis is however the most common and well-understood process. Autophagy can also cause programmed cell death (168). The role of autophagy in induction of cell death is paradoxical, as its primary function appears to protect cells during states of stress and nutrient deprivation (98). However it is clear that when autophagy is excessive, it can lead to a form of programmed cell death (168). Several anti-inflammatory cytokines like TGF- β (306) IL4, and IL13 (317) are able to induce apoptosis in immune cells. A potential mechanism by which ARBs can induce cell death is by blocking AT1R (102), a pro-survival pathway, and hence increasing activation of the proapoptotic AT2R receptors (164). Of the ARBs, the association of telmisartan with cell death via apoptosis is the most reported (64; 147; 216; 299). Telmisartan's ability to induce cell death relative to the other ARBs can be explained by the many other proapoptotic pathways it has been shown to activate. The pathways activated by telmisartan, which can potentially lead to cell death, include the PPAR γ pathway, (76; 321) the PPAR δ , (299) the AMPK pathway (214) and autophagy (269).

In cultured microglia, using both the LDH and MTS assays, we showed that telmisartan in contrast to all the other ARBs at 100 μ M can reduce viability of both primary microglia and BV2 cells. Many of the other ARBs did actually reduce LDH release from the cultured microglial cells. Induction of cell death by telmisartan was dose dependent and was maximal at 100 μ M within 12 hours of treatment. Cell death of BV2 cells due to telmisartan was confirmed using trypan blue exclusion assay. Possible mechanisms of

telmisartan induced cell death in microglia include apoptosis and autophagy as telmisartan has previously been shown to be proapoptotic (147) and we show in this study that telmisartan also induces autophagy in microglia. Treatment of BV2 cells with 3-MA, an early inhibitor of autophagy, partially reduced LDH release by telmisartan. Also treatment with the pan-caspase inhibitor Z-VAD-FMK, an apoptosis inhibitor similarly reduced telmisartan-mediated release of LDH from BV2 cells. These data suggest that telmisartan may be inducing apoptosis and autophagy mediated cell death in microglia.

Cell death can induce or mitigate inflammation. Cell death by necrosis or necroptosis is associated with the release of DAMPs which can incite inflammation (318). On the other hand, programmed cell death by apoptosis has many physiological roles including inhibition of inflammation (200) and regulation of embryonic development (30). Death by apoptosis therefore does not incite inflammation (109). Indeed, induction of apoptosis is thought to be associated with release of anti-inflammatory mediators and apoptosis of inflammatory cells is a therapeutic aim in inflammatory diseases like asthma and inflammatory bowel disease (200; 318). The inflammatory effect of cells that die by autophagy is not clearly understood (318). However the predominantly anti-inflammatory role played by induction of autophagy may suggest that cell death of immune cells like microglia by autophagy may be anti-inflammatory.

Thus far, it is not known whether reduction of microglial cell viability contributes to the anti-inflammatory and neuroprotective effects described so far for telmisartan. However, it is possible that telmisartan could prevent excessive microgliosis in the CNS by inducing microglial apoptosis and reducing proliferation to mitigate excessive neuroinflammation. In the first paper, the observation that the expected proapoptotic effect

of TGF- β was compromised in activated microglia raises the possibility that molecules like telmisartan could be used to augment such pathways to therapeutic ends. The concept of reducing the population of microglia for potential therapy was tested by Elmore *et al*, when they temporarily depopulated the CNS of about 99% of microglial population using a colony stimulating factor 1 antagonist and observed no significant detrimental effects (74). Another study using liposomal chlordonates reduced hippocampal microglial population by 40% without inducing neuronal cell loss (72). It will be interesting to see how temporarily eliminating microglia will be beneficial in neurodegenerative disorders.

In this study, we observed increase in LDH release with telmisartan at doses starting from 10 μ M indicating increased leakage of cytoplasmic content into the culture medium. More overt cell death was observed at doses of 50 and 100 μ M of telmisartan. Cell death by apoptosis due to telmisartan has been reported at a lower dose of 1 μ M in ovarian cancer cells (146). Although telmisartan has been shown to have toxic effects in several cell types, it has also been shown to be neuroprotective at 20 μ M in vitro (279). It is unclear whether higher doses of telmisartan would affect neuronal survival. This implies that telmisartan may have differential effects on cell death in different cells and contexts, which may be dose dependent. Differential induction of death therefore could be exploited, determining doses that can limit microglial population but be protective of neurons in CNS disease. It will also be necessary to determine whether there are doses of telmisartan at which significant neuronal death could occur as the ability to induce death by telmisartan presents a potential path to detrimental effects.

TELMISARTAN AS A POTENTIAL THERAPY FOR CHRONIC NEUROINFLAMMATION AND NEURODEGENERATION

In this study, we have shown that telmisartan can effectively reduce neuroinflammation. Activation of AMPK and induction of autophagy by telmisartan serve as an important prometabolic function which we have demonstrated in microglia. AMPK activation and induction of autophagy are similarly used by the prometabolic drug metformin in diabetes mellitus (275) implying that telmisartan could combine prometabolic effects with strong anti-inflammatory properties in treatment of neuropathology. It would however have to be determined whether telmisartan's protective effects of neurons is dependent on AMPK activation and induction of autophagy. Although we detected signs of toxicity on microglia with treatment of telmisartan, we believe it could be a beneficial mechanism in CNS pathology microglial cell death could be neuroprotective. None the less it would be worth taken due cognizance of the death inducing effect of telmisartan if the need arises for high doses in order to minimize potential detrimental effects. Telmisartan has been used extensively in clinical practice and its therapeutic index is very high (238) thus reducing worries of toxicity in treatment.

CONCLUSION

In this thesis, I was seeking to understand molecular mechanisms that mediate chronic neuroinflammation and also assess the use of ARBs in treatment of neuroinflammation and possibly neurodegenerative disorders. My first paper assessed the effect of microglia activation in inhibition of TGF- β signaling and begun to explore molecular pathways involved. I showed that the NF- κ B pathway is critical in inhibition of TGF- β signaling in activated microglia. My second paper mainly showed that telmisartan induces autophagy and cell death in microglia as a new addition to the literature and these may be in part contributing to its anti-inflammatory properties. With respect to microglia, TGF- β and telmisartan appear to have similar effects as they both reduce microglia activation and viability and are both directly neuroprotective. It could be assessed in further studies how these two systems interact and how that interaction could be exploited for therapeutic ends. Although studies in the kidney have shown some antagonism of the ARB telmisartan to TGF- β signaling (319), these interactions are generally context specific and therefore the interaction between TGF- β signaling and telmisartan would have to be independently assessed in microglia.

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