PLASMA ALDOSTERONE LEVELS INCREASE AFTER CEREBRAL ISCHEMIA, AND MINERALOCORTICOID RECEPTOR ANTAGONISM AT THE TIME OF REPERFUSION REDUCES INFARCT SIZE; THE ROLE OF MINERALOCORTICOID RECEPTOR ANTAGONISM ON THE INFLAMMATORY RESPONSE POST-STROKE

By

Carla Maria Dams Ramos

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ABSTRACT

Stroke is a leading cause of death and disability, and the available therapies are limited. Stroke stimulates inflammatory mechanisms that exacerbate the damage caused by cerebral ischemia. Interestingly, inflammation is linked to the effects of aldosterone and mineralocorticoid receptor (MR) activation in different organ systems. Previous studies show that chronic MR antagonism before cerebral ischemia improves the outcome of stroke, and MR activation worsens it. The aim of this study was investigate the hypothesis that plasma aldosterone levels increase after cerebral ischemia, and MR antagonists administered post-stroke, will reduce the infarct size by mediating a shift in the phenotype of the immune cells in the brain from pro- to anti-inflammatory. I induced transient focal cerebral ischemia using the middle cerebral artery occlusion technique, and the MR antagonist spironolactone was administered at the time of reperfusion. Plasma aldosterone levels were measured by ELISA, and inflammatory marker expression was analyzed by qRT-PCR. My results indicate that plasma aldosterone levels increase rapidly after the induction of cerebral ischemia in Wistar Kyoto (WKY) rats but not in stroke-prone spontaneously hypertensive rats (SHRSP). MR antagonism post-stroke reduced infarct size in both strains. In SHRSP, but not in WKY rats, MR antagonism increased the expression of anti-inflammatory microglia/macrophages (MG/Mϕ) and cytokines, which may be positive for stroke outcome. These results suggest that aldosterone post-stroke exacerbates the injury caused by cerebral ischemia and that MR antagonists are a potential drug for the treatment of stroke.

To my loving and supportive husband Alex,

who brought meaning and joy

to this chapter of my life.

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INTRODUCTION

Statement of the Problem

Stroke is a cerebrovascular accident and the second leading cause of death in the world. According to the World Health Organization, annually 15 million people suffer a stroke, and 5.5 million stroke victims die. Stroke is also the leading cause of disability in the U.S. [1]. The vast majority of stroke survivors face life-long disability and devastating consequences to their quality of life that include impairment of language, memory, and movement. Approximately 50% of stroke survivors will have permanent disability, placing a burden on their families and caregivers. To make this problem worse, despite the billions of dollars invested to research stroke therapies, treatment options are still very limited. The only drug approved to treat stroke is the recombinant human tissue plasminogen activator, also known as rtPA. This is a serine protease that causes thrombolysis leading to reperfusion, which is the reinstatement of the blood supply to the affected area. However, because rtPA conveys a risk of hemorrhagic transformation, this treatment can only be administered to patients within 4.5 hours of the onset of ischemia [2]. This limitation means that only 4.5% of patients receive this therapy [3]. Some patients with large vessel occlusions that are not eligible for rtPA can be treated by mechanical thrombectomy, which is the recannulation of the occluded artery by physical removal of the clot. This involves insertion of an arterial catheter to the site of occlusion. Mechanical clot extraction

with retriever (e.g. MERCI retriever) or aspiration methods (e.g. Penumbra Aspiration Systems), have higher rates of successful reperfusion than thrombolysis [4]. However, high risk of complications like hemorrhage and the difficulty of the procedures make them a last resort intervention. Therefore, there is a great need for new and improved therapies for the treatment of ischemic strokes.

Substantial research has been conducted to develop neuroprotective strategies, but despite their promising results in pre-clinical studies, neuroprotective agents have not been effective in the human population. Subsequently, the stroke field has devoted greater efforts to stroke prevention, and reperfusion strategies post-stroke [5]. However, reperfusion itself can worsen the outcome of a stroke by exacerbating the effects of ischemia, leading to blood brain barrier (BBB) disruption [6], and further damage known as reperfusion injury [7]. Most stroke patients ultimately require stroke rehabilitation therapy for successful neurorecovery [1]. The human brain has a remarkable ability to adapt and recover functionality after injury. Therefore, rehabilitation helps patients with disabilities relearn physical and cognitive skills impaired by the stroke. Physical, occupational, and speech therapies are the foundations of stroke rehabilitation.

Cerebral Ischemia

Stroke is a cerebrovascular condition that is diverse in nature. Strokes are classified as ischemic or hemorrhagic depending on the underlying cause.

Ischemic strokes are the most common type with an incidence of 87%, and they are the type of stroke of interest in this study. An ischemic stroke occurs when a cerebral blood vessel is occluded, interrupting the supply of oxygen and glucose to the affected area of the brain. Cerebral arteries are most commonly occluded; although venous strokes occur, these are rare [8, 9]. Cerebral blood vessels can be occluded by an embolus (traveling particle), or a thrombus, which is usually a platelet deposit (blood clot) that forms in the vessel. Thrombi often originate from atherosclerotic lesions, which are accumulations of lipids, leukocytes, and smooth muscle cells on the vessel wall [10]. On the other hand, most emboli are dissociated thrombus material from cerebral and peripheral vessels. However, cardiogenic blood clots, air, cholesterol crystals, and cell lumps are all potential emboli that can occlude cerebral vessels [11, 12]. About 50% of ischemic strokes are attributed to large-artery atherothrombotic disease, 25% are lacunar strokes which occur in small intracranial arteries, 20% are caused by cardiac emboli, and 5% are attributed to various rare causes [13].

The brain is particularly vulnerable to ischemia because of its high metabolic rate and dependence on aerobic respiration. In addition, the brain does not have the ability to store energy as in other tissues, therefore even a momentary interruption of the blood supply can lead to permanent tissue damage, this is known as infarcted tissue and it is the direct result of necrosis, which is cell death due to exogenous factors or insults such as ischemia [14]. These cells die as a direct result of the lack of oxygen and glucose supply,

forming the ischemic core. Surrounding the core, there is a layer of affected cells that are severely impaired, but could be rescued under the right conditions. This layer is known as the penumbra; these cells may die or survive affecting the overall outcome of stroke. Cell death in the penumbra is the result of both necrotic and programmed cell death processes (apoptosis). Some post-stroke therapeutic interventions aim to rescue the cells in the penumbra and minimize the size of infarcted tissue. Minimizing the damage in the ischemic penumbra is imperative because final infarct volume impacts neurological outcome [15]. Although, progression of the ischemic penumbra and infarct maturation are influenced by multiple factors, some studies using immunohistochemical and imaging techniques report that completed infarct maturation can take up to 4 weeks after stroke [16]. Therefore, there is potentially a large window for post-stroke therapeutic intervention.

The Ischemic Cascade

Ischemic conditions and signaling molecules from affected cells trigger a complex series of biochemical reactions identified as the ischemic cascade, which leads to inflammation and neuronal cell death [14]. The term cascade is a misnomer, as the order of events after ischemia does not follow a linear sequence of reactions; instead they form a network of processes involved in cell dysfunction. A major component of this network is the complex inflammatory response observed after cerebral ischemia, which is the main focus of this thesis

and will be discussed in detail later.

After onset of ischemia, the high metabolic rate of the brain causes rapid depletion of adenosine triphosphate (ATP) from cells. The brain is composed of aerobic tissue that generates ATP using the mitochondrial electron transport chain; therefore, seconds after the onset of ischemia, ATP depletion causes failure of diverse cellular functions, including ATP-dependent ion pumps [17]. This leads to collapse of the membrane electrochemical gradient and depolarization. Depolarization leads Ca^{2+} influx and release of excitatory neurotransmitters, including glutamate, which is cytotoxic at high concentrations [18]. Studies have shown that oligodendrocytes, and the myelin they provide, are damaged by glutamate [19], while axonal processes are sensitive to Ca^{2+} accumulation [20]. Ca^{2+} influx also acts as a second messenger to activate an array of transcription factors, phospholipases, endonucleases, and proteases which compromise cell function and integrity [21] [22]. In addition, the influx of Na^{+}, Ca^{2+}, and Cl^{-} into the cell leads to brain edema [23].

Apoptosis also contributes to cell death; this is a cell-driven series of biochemical events and morphological changes that lead to the cells self-destruction. The morphological changes observed during apoptosis include the formation of spherical masses of chromatin within the nucleus, and the appearance of membrane–bound structures containing dark chromatin [24]. The main biochemical changes observed during apoptosis include the breakdown of

DNA into nucleosomal segments of about 200 base pairs [25], and the activation of the caspase system [26] which is a complex system of cysteine proteases essential for the execution of apoptosis. These enzymes are activated by second messenger molecules such as cytochrome *c*, which are released from the mitochondria and play a significant role in the activation of caspase-3 [27], a ubiquitous enzyme of apoptosis. Activation of caspase-3 by cytochrome *c* is necessary for the execution of apoptosis [14].

Several of the signaling mechanisms involved in the ischemic cascade contribute to an overproduction of reactive oxygen species (ROS). Complex I and III of the mitochondrial electron transport chain are major sources of ROS [28], including detrimental molecules such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). However, these free radicals are also generated by a number of cytosolic reactions mediated by the enzymes cyclo-oxygenase, lipo-oxygenase, and xanthine oxidase [29, 30]. ROS cause oxidative stress by damaging cellular structures, nucleic acids, and lipid membranes. Peroxidation, a biochemical indicator of tissue injury by ROS [31, 32], leads to apoptosis through activation of mitochondrial-derived products [33] and activation of the JNK 1/2 pathway via NMDA receptor-mediated extracellular Ca^{2+} influx [34]. Lipid peroxidation causes neurotoxicity by activating map kinases, PKC isoforms, and activating the JNK-C-jun/AP-1 pathway which induces caspase activity [33, 35-37]. Other ROS, like peroxynitrile, interfere with dehydrogenase activity in the Krebs cycle reducing pyruvate dehydrogenase and

alpha-ketoglutarate dehydrogenase activity [38], this decreases the mitochondrial transmembrane potential [38].

The components of the ischemic cascade that have been discussed are interrelated and can influence each other; they are also exacerbated by reperfusion, which is the reinstatement of blood supply to the affected area. Reperfusion increases the production of second messengers and prostaglandins, inflammation, and mitochondrial dysfunction as indicated by elevated ROS and opening of the mitochondrial permeability transition pore (MPTP) [39]. Reperfusion also provides oxygen influx for ROS generation, and contributes to the effects of the ischemic cascade leading to BBB disruption [6]. This leaves the central nervous system vulnerable to infection.

Inflammatory Response to Ischemia

Inflammation is a complex reaction by the immune system to noxious stimuli such as pathogens, irritants, or ischemia. It is necessary for combating infection and wound healing. However, chronic inflammation can be harmful and even deadly. For this reason, the inflammatory response is tightly regulated. The inflammatory response is initiated when leukocytes are mobilized from the blood to the site of injury. In the brain, the inflammatory response to ischemia mediates a rapid activation of microglia (MG), the resident immune cells. These are myeloid cells that differentiate from monocytes and migrate from the bone

marrow to the brain where they reside and monitor the environment. MG act as the first line of immune defense in the brain by destroying foreign matter using phagocytic and cytotoxic mechanisms [40].

MG respond to changes in homeostasis and signaling molecules such as cytokines, ATP, glutamate, prostaglandins, and ROS [40]. These molecules can induce activation, migration, and proliferation of MG. Studies indicate that MG are activated within minutes of stroke onset [41, 42], leading to the release of cytokines and inflammatory mediators [41, 43]. Hours to days later, there is infiltration of inflammatory monocytes from the periphery [44], homologous to MG, which are known as macrophages (Mϕ). In experimental models of stroke, Mϕ start infiltrating 24-48 hours after ischemia and reach a peak 7 days later [45]. Because MG and Mϕ are morphologically and functionally similar, after Mϕ infiltrate in the brain, they cannot be differentiated from MG [46, 47]. For this reason, in the brain MG/Mϕ are analyzed and referred to as the same type of inflammatory cells. Other inflammatory cells such as neutrophils and T lymphocytes infiltrate the brain. Neutrophils are the first leukocytes to enter the brain, beginning after 30 minutes [48], T lymphocytes infiltrate to a lesser extent and play a role in the later stages of ischemic injury [49].

Inhibiting the inflammatory response by immunoblockade or genetic deletion of adhesion molecules and cytokines necessary for the activation and recruiting of inflammatory cells reduces the damage caused by ischemia [48].

Although the inflammatory response observed after stroke can significantly exacerbate the damage caused by cerebral ischemia [46] [50], inflammatory cells seem to play a dual role after stroke [51, 52]. MG/Mϕ are not a uniform population of cells, instead, they express multiple phenotypes depending on their environment. The expression of different phenotypes depends on signaling molecules and cytokines that can induce classical or alternative activation of MG/Mϕ [12]. Classically activated MG/Mϕ, also known as M1 are considered to be pro-inflammatory, while alternatively activated MG/Mϕ or M2 play an anti-inflammatory role and are involved in wound healing and tissue repair [52]. The M1 phenotype is induced by cytokines such as TNF-α and interlukin (IL)-1β [53]. M1 MG/Mϕ can be cytotoxic and exacerbate tissue damage. They mediate neurotoxicity by producing NADPH oxidase derived ROS, [54] and pro-inflammatory cytokines [55] which damage nearby cells and potentiate leukocyte infiltration, BBB disruption and apoptosis [56]. The cytokines associated with the M2 phenotype include transforming growth factor β1 (TGF-β1), IL-4 and IL-10 [53, 55]. This phenotype is characterized as a debris scavenger; M2 MG/Mϕ promote angiogenesis and extracellular matrix remodeling for cell growth [57]. M2 MG/Mϕ cannot carryout antigen presentation or promote inflammation. M2 MG/Mϕ are unable to activate NF-κβ, a transcription factor that responds to noxious stimuli [58] and is necessary to induce the pro-inflammatory response mediated by M1 MG/Mϕ.

One recent study showed that polarization of MG/Mϕ post-stroke is highly dynamic. Hu *et al*. conducted a temporal characterization of MG/Mϕ phenotype by M1 and M2 marker analysis with quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) [52]. The results revealed that in the early stages of cerebral ischemia, MG/Mϕ predominantly express the M2 phenotype, but gradually convert to M1 phenotype in and around the penumbra. They found that mRNA expression of all the tested M2 markers, which included TGF-β1 and Arginase (Arg) 1, were predominant soon after stroke and peaked by day 3 post-stroke. However, levels of M2 markers begin to decline around day 7 post-stroke until they are minimal compared to M1 markers. In contrast, mRNA of common M1 markers such as the glycoproteins Cluster of Differentiation (CD) 16, 32, 86, and 11b, as well as inducible nitric oxide synthase (iNOS) gradually increased from day 3 post-stroke and remained elevated for 2 weeks. mRNA expression changes were supported by IHC and *in vitro* experiments. Using an *in vitro* model of ischemia by oxygen-glucose deprivation of cultured neurons, the authors showed that M1 MG/Mϕ or conditioned media from these cells intensifies induced neuronal death. In contrast, M2 microglia protected neurons against ischemia. These results suggest that suppressing the shift from M2 to M1 is a potential therapeutic strategy to reduce the damage caused by ischemia [52]. This thesis aims to investigate if MG/Mϕ polarization is a target of the effects of the hormone aldosterone.

Aldosterone and the Mineralocorticoid Receptor (MR)

Aldosterone is a steroid hormone with high affinity for the MR. It is synthesized in the zona glomerulosa (ZG; outer layer) of the adrenal cortex [59]. Aldosterone primarily acts in the collecting duct of the kidney to regulate blood pressure through water and sodium reabsorption. Activation of the MR in epithelial tissues leads to up-regulation of epithelial sodium channel expression, causing Na^+ and water retention [59]. As a consequence the increase in extracellular volume leads to an increase in blood pressure. The MR plays a major role in the regulation of renal physiological functions, but recent studies have revealed that MR activation and high levels of its ligand, aldosterone can contribute to the pathology of other organ systems. In the vasculature, for example, MR activation leads to inflammation and remodeling, increasing the risk of stroke [60, 61].

The chemical structure of aldosterone was identified in 1953, but it was not until the 1990's when researchers began to elucidate the hormone's pathophysiological effects in cardiovascular disease [59, 62]. Since then, different roles of aldosterone, including effects on the cerebral vasculature and risk of stroke keep emerging. The main objective of this thesis is to further this research and investigate if blocking the effects of aldosterone by antagonizing the MR after a stroke reduces the damage caused by cerebral ischemia.

The MR is a nuclear receptor that has equal affinity for mineralocorticoids and glucocorticoids such as cortisol (humans) or corticosterone (rodents) [63]. The MR is expressed in many different tissues including the kidney, heart, and brain regions such as the hypothalamus and hippocampus [64, 65]. However, tissues where the MR is specific to mineralocorticoids, including vascular smooth muscle cells, contain the enzyme 11-β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) [65-67]. This enzyme metabolizes cortisol and corticosterone to cortisone, which is unable to bind to and activate the MR. This protects the MR from being constantly activated because physiological glucocorticoid concentrations are 100 to 1000-fold higher than circulating aldosterone [68]. Therefore, deficiencies in 11β-HSD2 lead to arterial hypertension and hypokalemia, which normally reflect excessive MR activation due to elevated levels of mineralocorticoids, however patients with 11β-HSD2 deficiency present low levels of mineralocorticoid and high levels of glucocorticoids [69].

The MR belongs to the group of 3-ketosteroid receptors in the estrogen receptor-like subfamily. The NR3C2 gene encodes the MR protein, which consists of three regions: the N-terminal domain, the C-terminal domain, which is also the ligand-binding domain, and the DNA binding domain, which serves as a transcription factor [63]. Similar to other nuclear receptors, when the MR is activated by a ligand, it undergoes conformational changes, leading to its dissociation from the heat shock protein 90, and homodimerization. Upon translocation to the nucleus, the MR complex interacts with coactivators and

hormone response elements present in the promoter of some genes. This results in transcription of target genes [65]. Although the genomic effects of the MR are better characterized, there is evidence of non-genomic effects of the MR that lead to activation of second messenger pathways [65].

Spironolactone and eplerenone are MR antagonists that block the MR with high affinity. Spironolactone is a synthetic compound that acts predominantly as a competitive antagonist, and has been used clinically as a K^+-sparing diuretic mostly for patients with cardiovascular conditions like heart failure and resistant hypertension [62, 70].

Aldosterone Regulation

It is unknown what happens to aldosterone levels post-stroke. Aldosterone production is mainly regulated by the renin-angiotensin system in response to elevated [K^+] or low blood pressure. High [K^+] stimulates aldosterone production by depolarizing cells in the ZG and activating voltage-dependent Ca^{2+} channels [59]. On the other hand, the hormones involved in the renin-angiotensin system stimulate aldosterone synthesis when the juxtaglomerular cells in the kidney release the enzyme renin upon sensing low blood pressure [71]. Renin catalyzes the conversion of angiotensinogen released by the liver to angiotensin (Ang) I, which is metabolized into AngII. This is a potent vasoconstrictor that can also act on the cells of the ZG to stimulate aldosterone production [59, 71].

Although studies indicate that MR antagonism is beneficial, particularly in cardiovascular disease, even when aldosterone levels are not elevated [72], the literature suggests that cerebral ischemia has the potential to increase aldosterone production. There is evidence that the sympathetic nervous system (SNS) could mediate increased aldosterone production via the increase in nerve activity that occurs post-stroke [73]. The ZG in the adrenal cortex is innervated by SNS nerves that come from the adrenal medulla and outside the adrenal gland [74, 75]. Some of the nerves that reach the adrenal cortex are post-ganglionic and express tyrosine hydroxylase, the enzyme necessary for NE production [76]. In addition, *in vitro* studies have shown that electrical stimulation of the capsule and ZG causes NE release [76] and NE, by activating β_1-adrenoreceptors, stimulates aldosterone secretion [77, 78], and enhances AngII-stimulated aldosterone production [79]. Furthermore, studies show the steroidgenic acute regulatory (StAR) protein, which is required to transfer cholesterol to the mitochondria for steroid biosynthesis [79] increases 24 hours after MCAO [80], suggesting steroidgenic activity may be elevated. All this evidence suggests that after stroke, sympathetic nerve activity may increase plasma aldosterone levels.

In models of congestive heart failure, aldosterone augments the activity of the paraventricular nucleus of the hypothalamus, which is critical for regulation of sympathetic drive and contributes to the progression of the disease [81]. This study also showed that MR blockade decreases sympathetic nerve activity in rats

through direct actions in the brain [81]. In addition, patients with heart failure treated with MR antagonists have improved NE uptake and heart-rate variability [82]. This evidence raises the question of whether cerebral ischemia induces a system of positive-feedback regulation between aldosterone and the SNS, similar to other positive-feedback mechanisms observed in states of disease. Understanding the mechanism for the increase in aldosterone production could open up alternative therapeutic targets for the treatment of stroke.

Stroke Risk is Increased by the Effects of Aldosterone

Elevated plasma aldosterone is associated with stroke independently of its link with blood pressure. Patients with elevated plasma aldosterone have a higher risk of suffering a stroke [83]. Comparison studies between patients with primary aldosteronism and essential hypertension showed that patients with primary aldosteronism suffer more strokes despite having lower blood pressure [84]. In addition, cerebral ischemia induces upregulation of MR expression in the hippocampus and cerebral cortex where the MR is normally expressed, but ischemia also induces MR expression in brain regions that do not express the MR under normal conditions such as the striatum [85]. In this study using mice, the authors showed that a short duration of ischemia (only 20 minutes of tMCAO) remarkably increases MR expression in the ischemic brain and that the majority of MR-positive cells are astrocytes that migrate towards the ischemic area [85].

Previous studies published by the Dorrance laboratory have shown that activation of the MR aggravates the outcome of cerebral ischemia. In one study, inhibiting the effects of aldosterone by blocking the MR prior to stroke decreased damage caused by cerebral ischemia by 50% without affecting blood pressure [84]. Similar results have been obtained in studies conducted with mice; one study showed that long term MR antagonism prior to cerebral ischemia reduces infarct size. The authors argued this results from elevated levels of neuroprotective and angionenic agents, growth factors such as vascular endothelial growth factor (VEGF), and a reduction in ROS production around the infarcted region [85]. However, it is important to point out that increasing levels of VEGF has been shown to be detrimental because it causes an increase in BBB permeability [86]. Another study using mice showed that MR inhibition for 2 days prior to the induction of cerebral ischemia with the antagonist eplerenone reduces the infarct size and neurological impairments by improving cerebral blood flow in the penumbra and reducing oxidative stress [87]. This study suggests that beneficial effects of MR antagonism are linked to improving vascular function because treatment for 48 hours with spironolactone is unlikely to have an effect on slow processes such as vascular remodeling and angiogenesis. So far, all the beneficial effects of MR antagonism seem to be blood pressure independent. In addition, chronic MR activation by two different mechanisms corroborated that MR activation increases the size of infarct after cerebral ischemia [60, 88]. These studies reported a significant, but small increase in blood pressure. However, the MR antagonists were administered

long-term before onset of ischemia, limiting the clinical relevance of these studies. At this point, there is enough evidence suggesting that MR antagonism could be a strategy for the treatment of stroke to warrant further studies with clinically appropriate treatment regimes. Importantly, several of the components of the ischemic cascade like oxidative stress, inflammation, and apoptosis have been linked to the effects of aldosterone and MR activation in different organ systems like the heart and kidney [89, 90].

MR activation and Inflammation

There is evidence suggesting that cerebral ischemia induces an increase in plasma aldosterone levels soon after stroke [73, 91]. If this is the case, it is possible that MR activation after ischemia exacerbates the outcome of stroke by altering the immune response and ROS generation. MR activation and aldosterone are linked to inflammation; MR activation induces activation of transcription factor NF$\kappa\beta$. This enhances the expression of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein- 1 (MCP-1) [92, 93] which are involved in activation of M1 MG/MΦ. An *in vitro* study using cultured human peripheral mononuclear cells (MΦ precursor) showed that incubation with aldosterone increases TNF-α expression [94]. In addition, both *in vitro* and i*n vivo* studies have shown that spironolactone treatment blocks TNF-α release through a serum- and

glucocorticoid-mediated kinase-1-dependant pathway (sgk1), which is the main mediator of MR genomic effects in epithelial and smooth muscle cells [95, 96].

Recently, studies using MΦ specific MR knockout mice suggest that the MG/MΦ phenotype is partly controlled by the MR. Usher *et al*, 2010 demonstrated that efficient classical activation of MG/MΦ requires a functional myeloid MR both *in vitro* and *in vivo* [97]. This means that myeloid MR deficiency promotes inducers of alternative activation such as IL-4 to shift the inflammatory response towards a M2 phenotype. *In vivo* myeloid MR knockout mice showed increased mRNA expression of anti-inflammatory cytokines such as TGF-β, IL-4, and Arg1 in heart tissue, and a reduction in cardiac hypertrophy and vascular damage caused by nitric oxide synthase inhibitor, *N*G-nitro-l-arginine methyl ester (L-NAME) and AngII administration [97]. Importantly, studies of cerebral ischemia using the myeloid MR knockout mice show that MG/MΦ in the infarct are phenotypically similar to alternatively activated MG/MΦ and infarct size is reduced [98, 99]. These suggest that the MR plays a role in the immune response observed post-stroke and spironolactone treatment at the time of stroke could potentially have the same effect that MR knockout on shifting the inflammatory phenotype. My study aims to investigate if MR inhibition with the antagonist spironolactone after the onset of ischemia has the potential to reduce infarct size by shifting the inflammatory response from pro- to anti-inflammatory phenotype. For this purpose the main hypothesis of this thesis is that: *Plasma aldosterone levels increase after cerebral ischemia, and MR antagonist,*

administered post-stroke, will reduce the infarct size by mediating a shift in the phenotype of the immune cells in the brain from pro- to anti-inflammatory.

Choice of Models

Stroke-prone spontaneously hypertensive rats (SHRSP) have been widely used to study spontaneously occurring hemorrhagic strokes and induced ischemic strokes. This strain was developed by the selective breeding of spontaneously hypertensive rats (SHR), which originated from Wistar Kyoto rats (WKY). The WKY is considered a normotensive control for SHR and SHRSP. The SHRSP shows elevated blood pressure from 6-weeks of age, malignant hypertension by 12 weeks, and as they age they can show stroke symptoms like circling and limb weakness [100, 101]. The SHRSP have been used to study spontaneous strokes, often, as the result of salt-loading and hypertension [102]. This high salt or stroke-prone diet induces cerebral hemorrhages, cortical infarcts, and small subcortical infarcts [101]. The latter resemble lacunar infarcts (micro-bleeds in brain white matter) in humans [103]. The subcortical infarcts suffered by the SHRSP are not only the consequence of elevated blood pressure. By developing consomic strains, Carswell *et al.* demonstrated that the incidence and magnitude of infarcts are independent of blood pressure and dependent on the genotype [104]. In addition, immunohistochemistry analysis of the progressive pathological changes that occur in the neurovascular unit of SHRSP rats showed that endothelial dysfunction, BBB impairment, and chronic

inflammation play contributing roles in the development of hypertension and stroke [105]. Although, SHRSP have proven to be an efficient model of spontaneous stroke, most commonly SHRSP are used to produce large middle cerebral artery (MCA) territory infarcts by MCA occlusion (MCAO). SHRSP have larger infarcts than WKY rats when ischemia is induced experimentally. In this study, I chose to study SHRSP to better understand the role of inflammation in the pathology of cerebral ischemia. In addition, because hypertension is a risk factor for stroke, the SHRSP provides a model with a co-morbidity, and comparing SHRSP to WKY rats allowed me to validate that MR antagonism post-stroke has beneficial effects on normotensive rats. The SHRSP used in this study were housed and fed identically to WKY rats and the SHRSP did not receive a high salt diet. However, the SHRSP and WKY rats used in this study were 12 weeks old, which is an age when SHRSP rats have developed hypertension.

EXPERIMENTAL DESIGN

This thesis consists of two specific aims that address the question presented in the central hypothesis: *Plasma aldosterone levels increase after cerebral ischemia, and MR antagonist, administered post-stroke, will reduce the infarct size by mediating a shift in the phenotype of the immune cells in the brain from pro- to anti-inflammatory.*

Aim 1: To test the hypothesis that plasma aldosterone levels increase after ischemic stroke and that MR antagonism at the time of reperfusion reduces the infarct size.

Aim 2: To test the hypothesis that MR antagonism improves stroke outcome by increasing the expression of anti-inflammatory cytokines as measured by qRT-PCR.

METHODOLOGY

Animals

All animal procedures were followed in accordance with Michigan State University animal use guidelines. A total of 53 SHRSP and WKY rats were used in this study. Eighteen SHRSP were obtained from the breeding colony

maintained by our laboratory at Michigan State University. Thirty-eight WKY rats were purchased from Harlan (Indianapolis, IN) at 11 weeks old, to allow one week of transport recovery before surgery. Aldosterone was measured in male WKY rats with femoral artery catheters that underwent 3 hours of tMCAO (Stroke; n=8) or sham surgery (SHAM; n=4). Aldosterone was also measured in SHRSP and WKY rats where blood was collected by cardiac puncture. This included, a group of intact and untreated SHRSP (SHRSP_SHAM; n=6) and WKY rats (WKY_SHAM; n=6) used as controls, a group of SHRSP (SHRSP_tMCAO+Control; n=6) and WKY rats (WKY_tMCAO+Control; n=6) that were only administered 200 ul of DMSO intraperitoneally (i.p.) as vehicle, and finally a group of SHRSP (SHRSP_tMCAO+Spir; n=6) and one group of WKY rats (WKY_tMCAO+Spir; n=6) were administered spironolactone at the time of reperfusion.

For infarct size quantification and inflammatory marker analysis rats were randomly assigned to the following groups: WKY: (WKY_MCAO+Spir; n = 11) or vehicle (WKY_MCAO+Control; n = 11). SHRSP: (SHRSP_MCAO+Spir; n = 5) or vehicle (SHRSP_MCAO+Control; n = 6).

The MR antagonist spironolactone (25 mg/kg b.w.) was diluted in 200 ul of the organic solvent dimethyl sulfoxide (DMSO; used as vehicle) immediately prior to use. Spironolactone was administered i.p. to the treatment groups of SHRSP and WKY rats right before removal of the monofilament to facilitate reperfusion.

Transient Middle Cerebral Artery Occlusion (tMCAO)

This method was previously described in Longa *et al* [106]. Rats were anesthetized with isoflurane and body temperature monitored with a rectal temperature probe to be maintained at 37°C with a heating pad. To achieve MCA occlusion, the carotid artery was exposed, the thyroid and lingual arteries were cauterized, and the external carotid and pterygopalatine arteries were ligated. The MCA was occluded by placing a 3.0 monofilament (Doccol, Sharon, MA.) through the internal carotid artery, and advancing it into the MCA. Occlusion of the MCA was verified by a drop in blood flow in the region supplied by the MCA as measured by pin-point laser Doppler. After MCAO, isoflurane administration was stopped to allow the rats to wake up. In WKY rats, MCAO was maintained for 3 hours, the occlusion time was limited to 1 hour in SHRSP. The rats were checked for neurodeficit during this time. The rats were then re-anesthetized, and the monofilament was removed. The rats were euthanized either 48 or 144 hours after the initial induction of ischemia.

Infarct Size Quantification

SHRSP and WKY rats underwent tMCAO (1 and 3 hours respectively). They were treated with either spironolactone or vehicle at the time of reperfusion as described above. The rats were euthanized, 48 hours after the onset of ischemia, under anesthesia and the brains were collected. Brains were coronally

sectioned in five 2 mm thick slices. The 4th slice from the frontal pole was snap-frozen in liquid nitrogen for mRNA expression analysis. The rest of the slices were stained with 2,3,5-triphenyltetrazolium chloride (TCC). The infarct volume was obtained using the formula of Swanson *et al* [107] (shown below), and is expressed as the percentage of the infarcted hemisphere to take cerebral edema into account. The slices were scanned and measurements were taken digitally using ImageJ (version 10.2). The 3rd slice was scanned front and back to account for the loss of the 4th slice from the equation.

The Swanson equation for measuring brain infarct volume:

$$\%\ I = 100 \times ((Vc - V_L) / Vc)$$

Where,

% *I* = Percent of hemisphere infarcted

Vc = Volume of normal tissue in the structure of the control hemisphere

V_L = Volume of normal tissue in the structure of the infarcted hemisphere

Measurement of Plasma Aldosterone Levels

Catheter Implantation

Femoral artery catheters (SAI, Lake Ville, IL) were implanted 2 days prior to tMCAO or sham surgery using the method described in Jespersen *et al.* [108]. Catheters were flushed with 200 ul of heparanized saline (100 U/ml) daily.

ELISA

To measure plasma aldosterone in SHRSP and WKY rats, an ELISA assay (Cayman Chemical, Ann Arbor, MI.) was used. Blood was collected either by cardiac puncture at the time of sacrifice or in conscious rats through a femoral artery catheter. Blood was centrifuged at 1000 rpm for 10 minutes to isolate plasma. Blood samples were collected before surgery and at the following post-stroke time points: 6, 24, 48, and 144 hours.

Analysis of Inflammatory Markers mRNA Expression

Real-Time PCR (qRT-PCR)

Total RNA was extracted from the ischemic hemisphere of SHRSP and WKY rats that underwent tMCAO and were treated with either spironolactone or vehicle. RNA was extracted using the Ambion® *mirVanaTM* PARIS Kit (Invitrogen, Grand island, NY), and reverse transcribed with either qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) or SuperScript® VILO™ (Life Technologies, Grand Island, NY) Specific TAQMAN primers were obtained from Applied Biosystems (Life Technologies, Grand Island, NY). The following inflammatory markers were analyzed: General MG/Mϕ markers; CD68 and intracellular adhesion molecule 1 (ICAM-1); M1 MG/Mϕ markers and pro-inflammatory cytokines; TNF-α, and MCP-1; M2 MG/Mϕ markers and M2 phenotype inducers; TGF-β1, Arg1. mRNA expression of each of the

inflammatory markers was normalized to expression of the housekeeping gene β-2-microglobulin (β2M). The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression from the qRT-PCR experiments [109].

Statistical Analysis

All data are presented as means ± standard error of the mean (SEM). For the comparison of two groups the appropriate Student's t test was used. In all cases, a *p* value of 0.05 or less was considered significant.

Plasma aldosterone concentration was quantified using a standard curve, and reported as concentration in picograms per milliliters (pg/ml). For aldosterone concentration analysis over time, a two-way repeated measures ANOVA was performed, and post-hoc testing using Bonferroni's procedure at each time point was performed to correct for multiple comparisons (Graph Pad Prism 5). For data analysis of aldosterone concentration in groups subjected to cardiac puncture, a One-way ANOVA with post-hoc testing using the Bonferroni's procedure was performed to analyze differences within treatment of SHRSP groups. For WKY data analysis within treatments, a One-Way ANOVA Kruskal-Wallis Test was performed with Dunn's post-hoc testing to correct for multiple comparisons with unequal variances.

RESULTS

Aim 1: To test the hypothesis that plasma aldosterone levels increase after stroke and MR inhibition at the time of reperfusion reduces infarct size.

Experiment 1: Plasma aldosterone concentration analysis after cerebral ischemia.

Experiment 2: Effect of MR antagonism at the time of reperfusion on infarct size.

Results of Experiment 1:

Analysis of plasma aldosterone levels in WKY rats with femoral artery catheters that underwent either tMCAO or sham surgery

Plasma aldosterone increased after cerebral ischemia in WKY rats with femoral artery catheters. Plasma aldosterone was significantly elevated 6 and 48 hours after induction of cerebral ischemia compared to shams. At 48 hours post-stroke plasma aldosterone was double that in shams (Figure 1). At 24 and 144 hours post-stroke aldosterone was also elevated, but this was not statistically different from the sham group, possibly due to the slight increase in aldosterone observed in shams. This increase could be due to the catheterization surgery itself. However, when plasma aldosterone levels at 24 and 144 hours were

compared to basal levels in tMCAO rats, they were both significantly increased (24 hours post-tMCAO: p value<0.0001; 144 hours post-tMCAO: p value<0.05; compared to Basal tMCAO). In contrast, plasma aldosterone levels did not increase in sham rats at any time compared to basal levels.

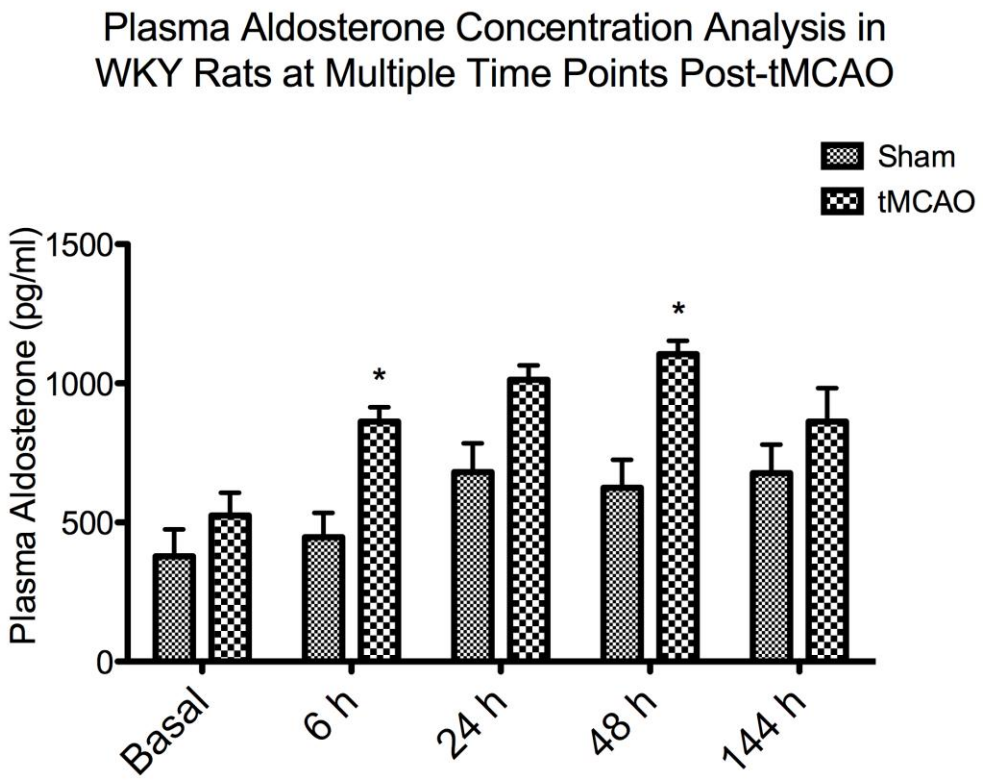

Figure 1.

Plasma aldosterone concentration analysis post-tMCAO in WKY rats with femoral artery cathters. Aldosterone was measured in WKY rats that underwent 3 hour of tMCAO (n=8) or sham surgery (n=4). Blood samples were collected before ischemia (Basal) and at 6, 24, 48, and 144 hours after ischemia. Plasma aldosterone concentraion (pg/ml) increased in WKY rats subjected to tMCAO at 6 and 48 hours post-tMCAO (*=p< 0.05 ; tMCAO compared to Sham).

Analysis of plasma aldosterone levels from blood samples collected by cardiac puncture in SHRSP and WKY rats that underwent MCAO compared to sham rats

SHRSP

tMCAO did not change plasma aldosterone concentration in SHRSP rats compared to SHRSP-SHAM (Figure 2). However, spironolactone treatment at the time of reperfusion did increase aldosterone levels compared to vehicle treated SHRSP and SHRSP sham groups. Plasma aldosterone levels were not statically different between SHRSP and WKY sham rats.

WKY

Plasma aldosterone was dramatically increased at WKY 48 hours post tMCAO (Figure 2, p value < 0.001). Plasma aldosterone levels increased about 15-fold after 3 hours of tMCAO followed by 45 hours of reperfusion in WKY rats. In addition, treatment with spironolactone at the time of reperfusion (WKY_MCAO+Spir) did not change plasma aldosterone levels when compared to WKY_MCAO rats.

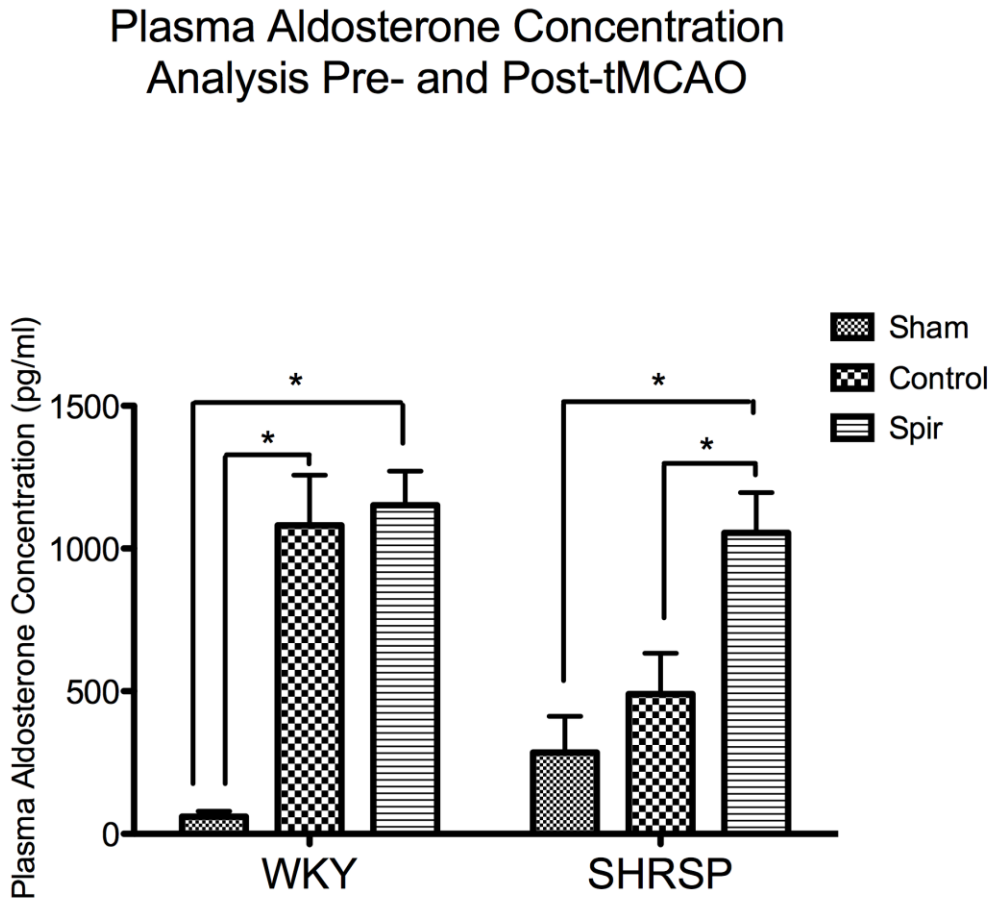

Figure 2.

Plasma aldosterone concentration analysis post-tMCAO in SHRSP and WKY. Aldosterone was measured in SHRSP and WKY rats that underwent 1 or 3 hour of tMCAO repectively and were treated with either spironolactone (Spir) or vehicle (Control). Intact and untreated rats of each strain were used for control (Sham). Plasma aldosterone concentraion (pg/ml) increased in WKY rats subjected to tMCAO and treated with Spir and Control compared to Shams (n=6 in each group; *=p< 0.05).

Results of Experiment 2:

Infarct size quantification in SHRSP and WKY rats

MR antagonism with spironolactone post-stroke reduces the size of the infarct caused by cerebral ischemia in SHRSP (Figure 3) and WKY (Figure 4) rats. Spironolactone reduced infarct size (average percentage of infarcted hemisphere) in SHRSP_tMCAO+Spir (n=5) by more than 30% compared to SHRSP_MCAO+Control (n=6) (p value < 0.01). In the WKY groups, similar results were observed; spironolactone treatment post-stroke significantly reduced infarct size in WKY_MCAO+Spir rats (n=11) compared to WKY_MCAO+Control; n = 11 (p value < 0.05).

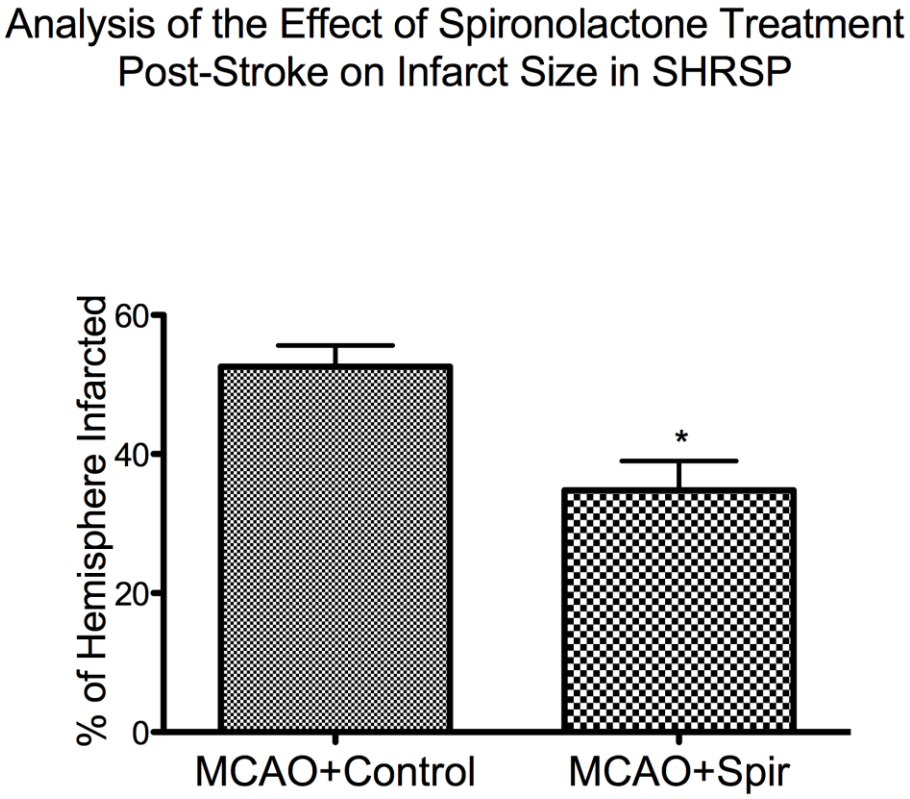

Figure 3.

MR antagonism with spironolactone at the time of reperfusion significantly reduced % of infarcted hemisphere in SHRSP that underwent 1 hour of tMCAO followed by 47 hours of reperfusion. The groups were treated with either spironolactone (n=5; tMCAO+Spir) or vehicle (n=6; tMCAO+Control) *= $p < 0.05$

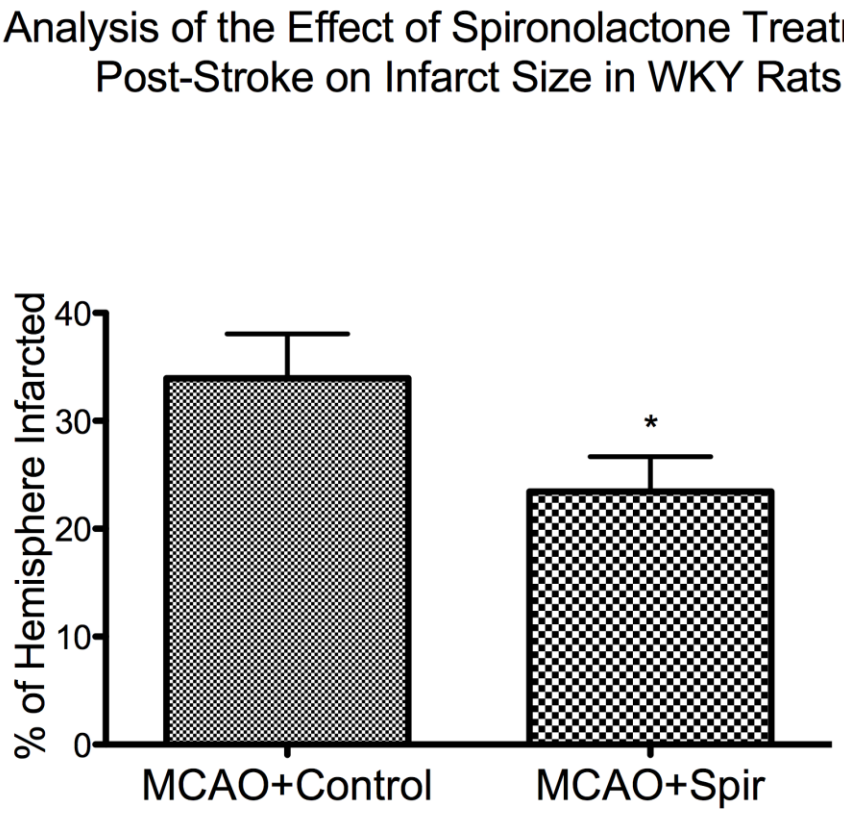

Figure 4.

MR antagonism with spironolactone at the time of reperfusion significantly reduced % of infarcted hemisphere in WKY rats that underwent 3 hour of tMCAO followed by 45 hours of reperfusion. The groups were treated with either spironolactone (n=11; tMCAO+Spir) or vehicle (n=11; tMCAO+Control) *= $p < 0.05$

Aim 2: To test the hypothesis that MR inhibition improves stroke outcome by increasing the expression of anti-inflammatory cytokines as measured by qRT-PCR.

Experiment 1: Analysis of inflammatory markers in SHRSP and WKY rats that underwent tMCAO and MR antagonism at the time of reperfusion.

Results of Experiment 1:

Analysis of Inflammatory marker mRNA expression in SHRSP and WKY rats

Figure 5 shows the fold increase in mRNA expression for general MG/MΦ markers CD68 and MG/MΦ cytokine ICAM-1. Groups treated with spironolactone or vehicle were compared for both strains to identify the effect of MR antagonist treatment post-stroke in the expression of inflammatory markers. SHRSP and WKY rats showed different patterns of marker expression in response to spironolactone treatment. CD68 was unchanged by spironolactone treatment in both SHRSP and WKY rats. ICAM-1 remained unchanged in the WKY_MCAO+Spir group compared to the control group. In contrast, in SHRSP_MCAO+Spir rats, expression of ICAM-1 mRNA is increased 3-fold compared to SHRSP_MCAO+Control (p value < 0.001). Figure 6 shows fold increase of pro-inflammatory cytokine, and M1 phenotype inducer, TNF-α. Spironolactone treatment did not change the mRNA expression of TNF-α in

SHRSP or WKY rats. Finally, mRNA expression of M2 MG/Mϕ phenotype and anti-inflammatory cytokines (TGF-β1, Arg1, and MRC-1) are shown in Figure 7. SHRSP and WKY rats exhibited different patterns of M2 marker mRNA expression; spironolactone treatment in WKY rats did not change the mRNA expression of any of the M2 markers. In contrast, mRNA expression of M2 phenotype marker Arg1 was significantly increased in SHRSP_MCAO+Spir rats compared to the SHRSP_MCAO+Control group (*p* value < 0.05). mRNA expression of M2 markers TGF-β1 and MRC-1 were not significantly changed.

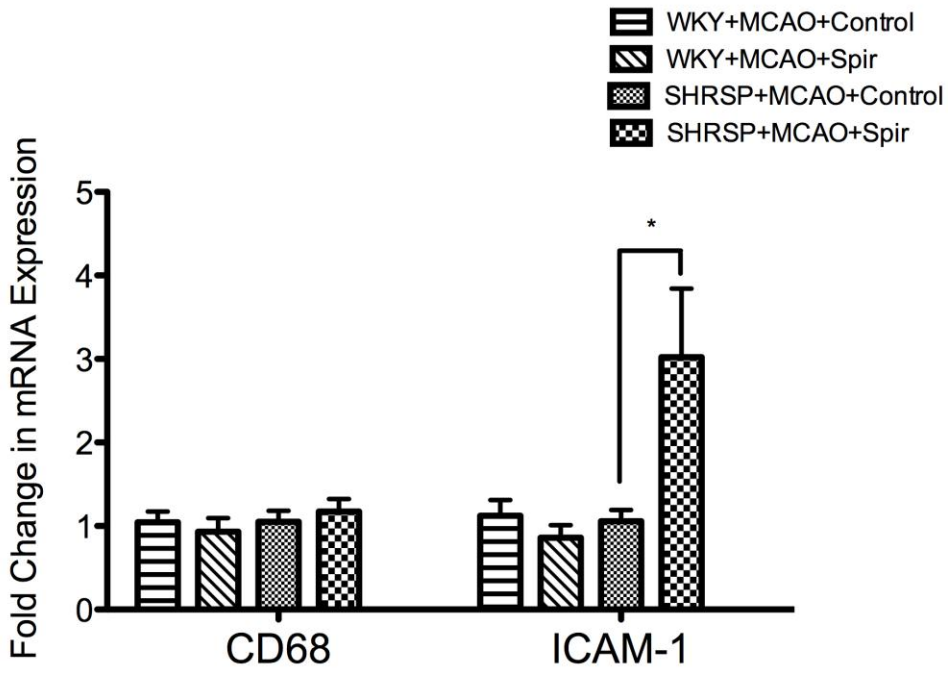

Figure 5.

MR antagonism with spironolactone at the time of reperfusion significantly increased mRNA expression of ICAM-1 in SHRSP comapred to SHRSP control. SHRSP and WKY rats underwent 1 or 3 hours of tMCAT followed by 47 or 45 hours of reperfusion respectively. Either spironolactone or vehicle treatment was administered at the time of reperfusion. The following groups were compared: WKY+tMCAO+Spir , n=7 and WKY+tMCAO+Control , n=7 ; SHRSP+tMCAO+Spir , n= 5 and SHRSP+tMCAO+Control , n= 6. *= $p < 0.05$

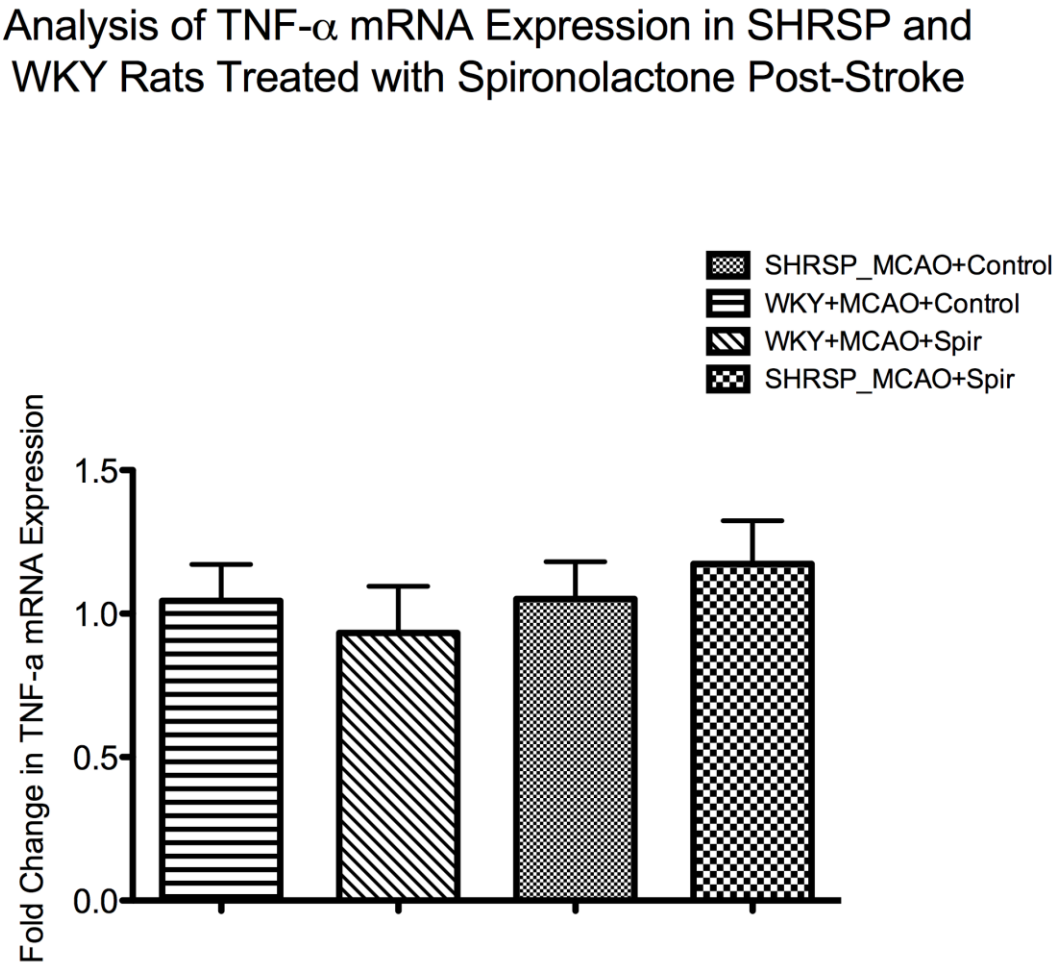

Figure 6.

MR antagonism with spironolactone at the time of reperfusion did not change mRNA expression of TNF-α in SHRSP and WKY rats. SHRSP and WKY rats underwent 1 or 3 hours of tMCAO followed by 47 or 45 hours of reperfusion respectively. Either spironolactone or vehicle treatment was administered at the time of reperfusion. The following groups were compared: WKY+tMCAO +Spir , n=7 and WKY+tMCAO+Control , n=7 ; SHRSP+tMCAO+Spir , n= 5 and SHRSP+tMCAO+Control , n= 6.

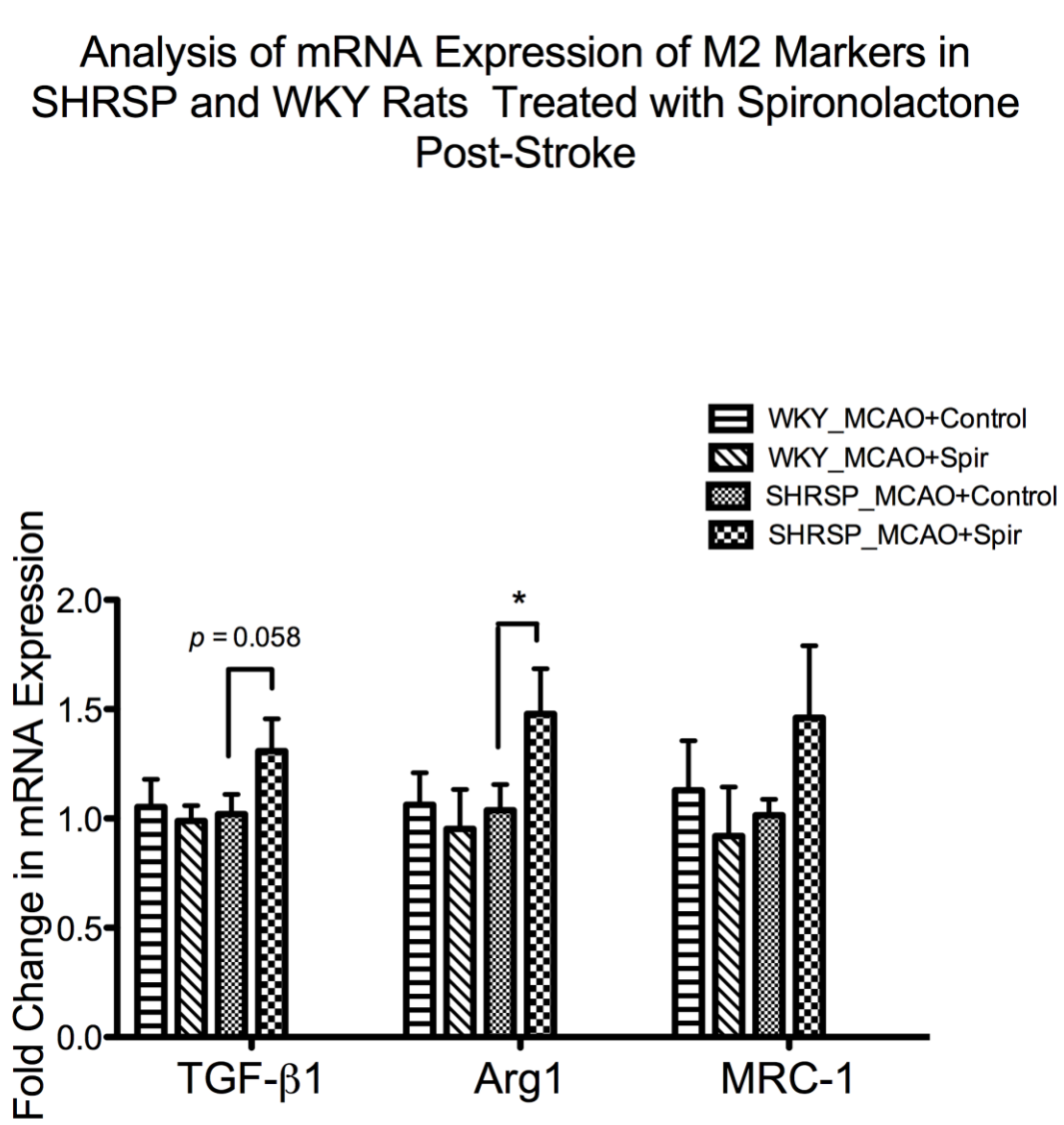

Figure 7.

MR antagonism with spironolactone at the time of reperfusion significantly increased mRNA expression of Arg1 in SHRSP compared to SHRSP control. The mRNA expression of M2 phenotype inducer TGF-β1 is slightly increased in SHRSP rats treated with spironolactone (*p* value = 0.058). SHRSP and WKY rats underwent 1 or 3 hours of tMCAO followed by 47 or 45 hours of reperfusion respectively. Either spironolactone or vehicle treatment was administered at the time of reperfusion. The following groups were compared: WKY+tMCAO+Spir , n=7 and WKY+tMCAO+Control , n=7 ; SHRSP+tMCAO +Spir , n= 5 and SHRSP+tMCAO+Control , n= 6. *= p <0.05

DISCUSSION

The results obtained in this study support parts, but not all, of my central hypothesis. Plasma aldosterone levels are increased by cerebral ischemia in WKY rats but not in SHRSP. However, MR antagonism at the time of reperfusion reduces infarct size in both strains studied. Finally, MR antagonism improves the outcome of stroke by shifting the phenotype of MG/Mϕ from pro- to anti-inflammatory in SHRSP, but not in WKY rats. This indicates that the mechanism of action for the beneficial effects of MR antagonism is different in the two strains and could depend on pre-existing or genetic factors.

Ischemia Increases Plasma Aldosterone Levels

My study showed that plasma aldosterone levels increase remarkably after the onset of ischemia. WKY rats with femoral artery catheters had elevated plasma aldosterone concentrations 6 hours after the onset of ischemia compared to sham operated rats. Aldosterone levels remained elevated up to 48 hours post-stroke. Surprisingly, aldosterone levels were not significantly higher at 24 hours post-stroke. This could be due to the slight increase in aldosterone observed in the sham-operated rats at 24 and 144 hours post-stroke. Although we do not know the exact mechanism, it is possible that stress and inflammation associated with surgery could mediate the increase in aldosterone levels after catheterization in the sham rats. For this purpose we wanted to investigate if

MCAO increased plasma aldosterone levels in the same manner in rats without femoral artery catheters. These blood samples were collected while the rats were under isoflurane anesthesia; however, this anesthetic has little effect on aldosterone levels [114]. We analyzed data from both SHRSP and WKY rats to determine if the increase in aldosterone levels was strain dependent. We previously used Sprague-Dawley rats and found that aldosterone levels increases post-stroke. This suggests that the increase in aldosterone levels is blood pressure instead of strain dependent. Moreover, it is possible that plasma aldosterone levels in SHRSP do not have the ability to increase any further if SNS activity is already elevated. In addition, we analyzed data from groups treated with MR antagonist spironolactone to determine if spironolactone treatment could increase aldosterone levels further. The results were very interesting; first of all, catheterization seems to increase aldosterone levels, WKY rats that did not undergo any treatment or surgery had much lower basal levels of aldosterone (about an 8-fold difference). This finding is potentially important when one considers that catheters are generally accepted as one of the best ways to collect blood for aldosterone analysis. Second, MCAO alone did not increase plasma aldosterone levels in SHRSP as observed in WKY rats. However, MR antagonism significantly increases plasma aldosterone levels in SHRSP. Finally, in contrast to SHRSP, MR antagonism in this group significantly increased plasma aldosterone levels. This was an interesting finding, and could indicate that aldosterone production increases to its maximal capacity after cerebral ischemia in WKY rats; therefore, MR antagonism cannot further

increase aldosterone levels. One way MR antagonism could increase the production of aldosterone is by mimicking a positive-feedback mechanism to the HPA axis. Differences in plasma aldosterone levels between the strains were surprising because the Dorrance laboratory had reported no differences between the strains [84]. It is important to mention that in this study the rats were given a fatal dose of pentobarbital, which could potentially increase aldosterone levels. Nevertheless, this is a controversial area. A different study reported that SHRSP have higher levels of plasma aldosterone than WKY rats [115]. These could be due to different diets, and blood sample collection methods, including methods of anesthesia.

Importantly, aldosterone levels increase dramatically during a critical period of cerebral ischemia resolution. Within 48 hours post-stroke onset, peripheral Mϕ infiltrate the brain and there is a widespread opening of the BBB [6, 14], which is highly detrimental for the outcome of ischemia and central nervous system health [116]. Therefore, MR activation could have negative effects on the outcome of cerebral ischemia through multiple pathways.

Although the mechanism for increased aldosterone production post-stroke remains unclear, some studies suggest ischemia can lead to increased aldosterone levels by stimulating sympathetic drive [117, 118]. One particular study showed that ischemic conditions in the heart have the potential to permanently activate central nervous system (CNS) nuclei that control

sympathetic outflow and increase norepinephrine release [118]. Similarly, there are other mechanisms that could lead to increased plasma aldosterone levels post-stroke, for example, the renin-angiotensin-system, which regulates aldosterone synthesis under normal circumstances. In addition, the HPA axis could also induce aldosterone production post-stroke via ACTH; since this hormone is also increased by ischemia [119]. Regardless of the responsible mechanism, finding that plasma aldosterone concentrations increase after cerebral ischemia raises questions and opens an opportunity to better understand the pathology of stroke. To the best of my knowledge, these are the first studies to show a marked increase in aldosterone with ischemia reperfusion injury. Increased aldosterone levels post-stroke could potentially affect other organ systems as well, particularly, the myocardium, where MR activation leads to structural remodeling [92].

Effect of MR Antagonism at the Time of Reperfusion on Infarct Size

To better understand the role of the MR in the outcome of cerebral ischemia I administered the MR antagonist spironolactone at the time of reperfusion. Previous studies have shown that chronic treatment with MR antagonists before stroke reduces infarct size, however, in this study I used a clinically relevant treatment paradigm to investigate the therapeutic potential of spironolactone. Interestingly, we found that MR antagonism post-stroke significantly reduces infarct size in both SHRSP and WKY rats compared to their

respective vehicle-treated controls. The duration of ischemia was increased to 3 hours in the WKY rats to produce a similar infarct size to the SHRSP. The differences in infarct size between the spironolactone-treated and control groups are not due to differences in cerebral blood flow; scanning laser Doppler (PIM3; Perimed, Stockholm, Sweden) measurements of pial artery blood flow showed no difference between the groups in SHRSP rats that underwent 1 hour of tMCAO followed by 23 hours of reperfusion (data not shown). However, since the scanning laser Doppler only measure flow in the pial arteries we do not know if there is a difference in blood flow in the sub-cortical regions or in the ischemic penumbra. The results from these experiments indicate that MR antagonism post-stroke is beneficial by intervening in pathways that aggravate stroke outcome, for example the inflammatory response, and the effects are not limited to the hypertensive population.

Effect of MR Antagonism Post-stroke on the Inflammatory Response

As previously discussed, several components of the ischemic cascade, including inflammation, have been linked to the effects of aldosterone and MR activation in different organ systems and conditions. The effect of spironolactone treatment on the post-stroke inflammatory response in SHRSP and WKY rats present interesting differences. MR antagonism at the time of reperfusion does not change the inflammatory response in WKY rats. There is a small but insignificant reduction in TNF-α with spironolactone treatment. It is possible that

the inflammatory analysis in WKY rats was underpowered and increasing the number of subjects could have led to the identification of a significant reduction in TNF-α expression. On the other hand, when spironolactone treatment is administered in SHRSP at the time of reperfusion, the expression of general MG/MΦ marker CD68 remains unchanged while ICAM-1 is significantly increased. ICAM-1 is required for MΦ to infiltrate into the tissue; therefore, this increase in ICAM-1 mRNA expression could indicate increased MΦ infiltration in the brain. This idea is supported by mRNA expression analysis of TNF-α, which is a pro-inflammatory cytokine that induces classical activation, and is unchanged by spironolactone treatment. In addition, the marker of M2 phenotype Arg1 is significantly increased in SHRSP treated with spironolactone. TGF-β1, an inducer of alternative activation shows a small increase after MR blockade, however, this was not significant different from SHRSP controls (*p* value = 0.0583). Similarly, although the M2 marker MRC-1 seems to be slightly increased by spironolactone treatment (*p* value = 0.09), this is not significant. It is possible that in these studies, statistical variability is high because we analyzed samples that contained normal and infarcted tissue; our findings might have been more conclusive if we had only analyzed tissue punches from the ischemic penumbra.

The inflammatory response has normally been considered to have detrimental effects in the resolution of ischemia. It leads to increased ROS production and apoptosis [46]. However, more recent evidence has shown that the inflammatory response can be beneficial and lead to tissue repair depending

on the phenotype exhibited by MG/Mϕ [97-99]. This study demonstrates that in SHRSP MR antagonism post-stroke has the potential to improve the outcome of cerebral ischemia by shifting the polarization of MG/Mϕ toward an alternative phenotype that leads to an anti-inflammatory response. In WKY rats, the beneficial effects of MR antagonism seem to be independent from the inflammatory response. The difference between strains can possibly be explained by the differences in duration of ischemia and timing of the treatment. Because we treated the rats at the time of reperfusion spironolactone was administered to WKY rats 2 hours later than in the SHRSP. Therefore, it is possible that spironolactone can only effectively modulate the inflammatory response within that one-hour window. Another possible explanation for the difference between strains is the evidence that SHRSP rats develop chronic inflammation in the absence of an ischemic insult, as evidenced by increased inflammatory cytokines and vascular lesions associated with chronic inflammation. Therefore, it is possible that the acute inflammatory response caused by ischemic conditions in WKY rats does not respond to MR blockade because there are other factors signaling the urgent state of injury.

The study on the temporal characterization of MG/Mϕ post-stroke recently published by Hu *et al*. indicates that the initial ischemia induces a “healthy” inflammatory response in the early stages of injury. However, within 48 hours after ischemic onset MG/Mϕ transitions to a “sick” phenotype which can worsen the outcome of the stroke [52]. It is possible that this transition is partly mediated

by the increase in plasma aldosterone observed after cerebral ischemia, Therefore, blocking the MR with spironolactone could be beneficial by prolonging the prevalence of M2 phenotype, increasing the potential for tissue repair and improving stroke outcome. Hu *et al*. subjected the mice to 1 hour of tMCAO; the same duration than the SHRSP in my study. This could explain my results in SHRSP, but not in WKY rats, are in accordance with those reported Hu *et al.* Further research is necessary to elucidate these gaps in our knowledge. However, the studies in this thesis are the first to demonstrate it is possible to use a clinically relevant treatment regime to alter the inflammatory response observed post-stroke in a rat model with a common co-morbidity for stroke such as hypertension.

LIMITATIONS

There were several limitations in the experimental design of this study. First of all, only one spironolactone dose, administration route, and treatment time was used for the study. It is possible that a different dose or time of administration would be more efficient in reducing infarct size and/or increasing the expression of anti-inflammatory markers and cytokines. Similarly, administration of the antagonist intravenously would be more precise and potentially more effective. In addition, this study only investigated the effects of MR antagonism in a model of ischemia/reperfusion injury. Therefore, we do not know if the same response would be observed in a model of permanent MCAO.

Another limitation is that the decrease in infarct size observed post-stroke is relatively small. Therefore, we do not know if spironolactone treatment would significantly improve neurological function. For this reason, neurological testing of the subjects before euthanasia would be insightful and imperative to assess the therapeutic potential of MR antagonism post-stroke. I did not investigate the mechanism by which MR antagonism mediates a shift in the inflammatory response. Finally, spironolactone, although it is an effective MR antagonist, is not MR specific. Spironolactone has been shown to also block the androgen receptor [120], which could potentially have an effect on the infarct size and the inflammatory response. Future studies should address these issues.

CONCLUSION

The studies conducted in this thesis highlight the therapeutic potential of the MR antagonist spironolactone for the treatment of cerebral ischemia. These studies indicate that the MR is involved in the pathology of cerebral ischemia and that increased plasma aldosterone levels post-stroke aggravate the injury caused by stroke. For this reason, a better understanding of the mechanisms involved in MR activation post-stroke is imperative. The complex nature of a stroke makes it unlikely that one therapeutic strategy will be a magic bullet for the treatment of stroke. MR blockade is a promising strategy because it might globally target multiple components of the ischemic cascade. Particularly interesting is the effect of MR antagonism on the inflammatory response observed post-stroke. A better

characterization of inflammatory cell phenotypes and function has suggested that the best strategy is not to suppress the inflammatory response, but instead to shift it towards a beneficial phenotype, which will contribute to tissue repair and healing. Spironolactone treatment post-stroke seems to have the potential to accomplish this. However, the complexity of the inflammatory response demands a better understanding of the pathways involved in MR mediation of inflammation, and we hope this study has helped spark interest in these questions.

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