Myeloid-Specific Deletion of the Mineralocorticoid Receptor Reduces Infarct Volume and Alters Inflammation During Cerebral Ischemia

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Background and Purpose—Mineralocorticoid receptor (MR) antagonists have protective effects in rodent models of ischemic stroke, but the cell type-specific actions of these drugs are unknown. In the present study, we examined the contribution of myeloid cell MR during focal cerebral ischemia using myeloid-specific MR knockout mice.

Methods—Myeloid-specific MR knockout mice were subjected to transient (90 minutes) middle cerebral artery occlusion followed by 24 hours reperfusion (n=5 to 7 per group). Ischemic cerebral infarcts were identified by hematoxylin and eosin staining and quantified with image analysis software. Immunohistochemical localization of microglia and macrophages was performed using Iba1 staining, and the expression of inflammatory markers was measured after 24 hours of reperfusion by quantitative reverse transcription–polymerase chain reaction.

Results—Myeloid-specific MR knockout resulted in a 65% reduction in infarct volume (P=0.005) after middle cerebral artery occlusion. This was accompanied by a significant reduction in activated microglia and macrophages in the ischemic core. Furthermore, myeloid-specific MR knockout suppressed classically activated M1 macrophage markers tumor necrosis factor-α, interleukin-1β, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, and interleukin-6 at the same time as partially preserving the induction of alternatively activated, M2, markers Arg1, and Ym1.

Conclusions—These data demonstrate that myeloid MR activation exacerbates stroke and identify myeloid MR as a critical target for MR antagonists. Furthermore, these data indicate that MR activation has an important role in controlling immune cell function during the inflammatory response to stroke. (Stroke. 2011;42:179-185.)

Key Words: inflammation ■ ischemia ■ macrophage ■ mineralocorticoid receptor ■ stroke

Mineralocorticoids can cause vascular inflammation and hypertension, which lead to vascular damage and remodeling.1 During ischemic stroke, mineralocorticoid receptor (MR) activation results in increased vascular damage and ischemia.2 Not surprisingly, several studies have shown that MR antagonists, at doses that do not alter blood pressure, are protective in rodent models of ischemic stroke. Treatment with the MR antagonist spironolactone was shown to reduce vascular damage and decrease mortality.3 Similarly, another MR antagonist, eplerenone, decreases superoxide production and reduces infarct volume in animal models of ischemic stroke.4 This indicates that MR blockade might have clinical potential as a therapeutic agent for stroke. However, the mechanisms of pharmacological control and, importantly, the cell type-specific actions of MR antagonists have not been identified and characterized.

In addition to its classical role in the kidney, MR has also been identified in other tissues, including the heart, brain, and inflammatory cells such as macrophages and microglia.5,6 In many of these cells, particularly brain and hematopoietic cells, the ligand for MR is thought to be glucocorticoids. MR has 2 high-affinity physiological ligands, mineralocorticoids such as aldosterone and glucocorticoids such as corticosterone, in rodents.7 Because glucocorticoids circulate at levels 100- to 1000-fold higher than mineralocorticoids, MR-binding sites are thought to be occupied by glucocorticoids in...
the absence of 11β-hydroxy-steroid dehydrogenase 2, which inactivates corticosterone to 11β-dehydrocorticosterone. Neurons and hematopoietic cells lack 11β-hydroxy-steroid dehydrogenase 2 and so the majority of MR molecules are predicted to be occupied by glucocorticoids.8

Inflammation has an important role in the pathogenesis of ischemic stroke. A reduction in immune cells, inflammatory cytokines, and adhesion molecules reduces stroke injury9,10 whereas increases in anti-inflammatory cytokines such as interleukin (IL)-10 and IL-1-receptor antagonist are protective during models of cerebral ischemia.11,12 Several strategies to reduce the damaging inflammatory response after ischemic stroke have targeted immune cells and immune cell recruitment. Decreasing neutrophil infiltration reduces infarct volumes and neuronal cell death in mice after focal cerebral ischemia.13 However, there was no neuroprotection found in clinical trials that tested agents that reduced neutrophil activity.13 Similarly, adhesion molecules are important for leukocyte trafficking and infiltration into ischemic regions, and the use of monoclonal antibodies against intercellular adhesion molecule-1 has been successful in animal models of ischemic stroke.14,15 Again, this treatment failed to translate to the clinical condition, but this was possibly due to the use of murine immunoglobulin. Targeting nuclear receptors that alter inflammation may be a viable alternative.

We have recently identified MR as a regulator of macrophage polarization, and deletion of MR from macrophages induces an alternatively activated macrophage phenotype, sometimes called M2, at the same time as suppressing the classically activated, M1, phenotype.16 Decreasing the M1/M2 ratio was associated with abrogation of L-arginine methyl ester/angiotensin II-induced cardiac and vascular hypertrophy, fibrosis, and inflammation. Myeloid MR is also important in DOCA/salt-induced cardiac fibrosis.17 Importantly, our previous work showed these effects to be independent of blood pressure-lowering and, rather, are proposed to be a result of MR control of macrophage activation. We therefore hypothesized that the neuroprotective effects of MR antagonists during cerebral ischemia are at least partially due to a modulation in myeloid cell response, particularly the M1/M2 polarization of macrophages and microglia. To test this, we examined the effects of myeloid MR knockout (MyMRKO) in a model of focal cerebral ischemia.

Figure 1. Effect of MyMRKO on blood pressure. Data represents the mean systolic pressure (A), diastolic pressure (B), and heart rate (C) of FC and MyMRKO mice during day and night cycles determined by implanted arterial pressure transducers. n=4 per group.

Methods

Mice
LysM-Cre mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). MyMRKO mice on a C57BL/6 background were bred by crossing homozygous floxed MR mice (provided by G. Schütz, German Cancer Research Center, Heidelberg, Germany) with homozygous floxed MR mice containing LysM-Cre (MR^{floxed};LysM-Cre×MR^{floxed}). MR^{floxed};LysM-Cre (knockouts) and littermate MR^{floxed} (floxed controls [FCs]) were used for all experiments. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 80-23) and were approved by the University Committee on Use and Care of Animals of the University of Michigan.

Middle Cerebral Artery Occlusion
Male MyMRKO mice weighing between 25 to 32 g were used. Middle cerebral artery (MCA) occlusion was performed using the intraluminal filament method as previously described.18 The mice were anesthetized with 1% to 3% isoflurane and a 6-0 silicon rubber-coated nylon monofilament (Docoll Corporation) was inserted into the right internal carotid artery. The right MCA was occluded for 90 minutes at which point the monofilament was removed and mice were allowed to recover.

Measurement of Infarct Volume
See supplemental methods (available at http://stroke.ahajournals.org).

Immunohistochemistry
See supplemental methods.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction
See supplemental methods.

Statistics
Data are presented as mean±SEM. Comparison of mean values between groups was performed using an unpaired, Student t test or by a 2-way analysis of variance with a Bonferroni posttest as indicated. P<0.05 was considered statistically significant.

Results

Myeloid-Specific MR Knockout
There are no obvious phenotypic differences in MyMRKO mice compared with FCs. Because MR is known classically to regulate blood pressure and this can affect stroke, we determined if MyMRKO affected blood pressure. We observed no significant change in baseline systolic and diastolic blood pressure between freely moving, unanesthetized MyMRKO and FC groups as measured by arterial pressure transducers monitored by radiotelemetry (Figure 1A–B). There is also no change in heart rate between the
groups (Figure 1C). This would indicate that differences in neurological outcome between the FCs and MyMRKO are unlikely to be related to blood pressure.

MyMRKO Reduces Infarct Volume

We examined the effect of MyMRKO on ischemic lesion size during focal cerebral ischemia. MyMRKO resulted in a significant reduction in infarct size 24 hours after a 90-minute transient occlusion of the right MCA. The infarct volume was determined in hematoxylin and eosin-stained serial coronal sections using Image J software and a significant decrease in ischemic infarct size was detected in MyMRKO sections (Figure 2A) relative to FCs (Figure 2B). Quantification of infarct volumes in serial coronal sections shows a significant reduction in MyMRKO (Figure 2C). The total infarct size of the ischemic hemisphere in the MyMRKO group was 11%, which was significantly less ($P<0.005$) than FCs, which had a total infarct volume of 32% (Figure 2D). This represented a highly significant 65% reduction in ischemic infarct volume in the MyMRKO group. No differences in pH, pO2, or pCO2 were detected before or during ischemia (Supplemental Table 1). Cerebral blood flow in the MCA territory was reduced to <50% baseline during ischemia, but no differences were seen in perfusion between FC and MyMRKO mice.

Activation of Myeloid-Derived Microglia/Macrophages After MCA Occlusion

After MCA occlusion, there were no differences in the number of microglia in the nonischemic, contralateral hemisphere between FC and MyMRKO groups (Figure 3A). There

Figure 2. Quantification of infarct volume after transient cerebral ischemia. Representative photographs of MyMRKO (A) and FC (B) showing a reduced infarct size in the MyMRKO group. Quantification of infarct volume in serial coronal sections of FC and MyMRKO mice (C) and quantification of total ischemic infarct size in whole brain hemispheres (D) also showed a significant reduction in infarct size in the MyMRKO group. n=5 to 7 per group. **$P<0.01$, ***$P<0.001$, Bonferroni posttest.

Figure 3. Immunohistochemical analysis of activated microglia and macrophages after MCA occlusion. Representative photomicrographs of nonischemic contralateral (Contra) and ischemic ipsilateral (Ipsi) regions from coronal sections of FC and MyMRKO (A). Quantification of immunoreactive Iba1$^{+}$ cells in the ischemic core showed a significant decrease in macrophages/microglia in of MyMRKO mice (B). n=5 to 7 per group.
was a robust increase in Iba1+ cells in the ischemic, ipsilateral core when compared with the nonischemic, contralateral hemisphere in FCs, indicating an increase in microglia activation and/or macrophage recruitment. However, this response was reduced in MyMRKO mice. Quantification of Iba1+ cells/field showed a statistically significant reduction ($P=0.018$) in microglia/macrophages in MyMRKO in the ischemic core (Figure 3B). A regional comparison of Iba1+ cells shows that significant differences in microglia/macrophages are largely confined to the subcortical basal ganglia (Table), which is within the ischemic core.

MyMRKO Alters the Inflammatory Response to Stroke

We have demonstrated previously that myeloid MR controls macrophage polarization and alters the inflammatory response during cardiac inflammation and fibrosis. To determine whether MyMRKO alters the inflammatory response to ischemic stroke, we measured the expression of classical and alternative macrophage markers 24 hours after MCA occlusion using quantitative reverse transcription–polymerase chain reaction. There is a strong induction in proinflammatory cytokines in the ischemic hemisphere of the FC group. However, MyMRKO demonstrated profound suppression of classically activated M1 markers tumor necrosis factor-$\alpha$, IL-1$\beta$, monocyte chemoattractant protein-1, and macrophage...

<table>
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<tr>
<th>Anatomic Region</th>
<th>Iba1+ Cells/Field</th>
<th>FC Mean±SE</th>
<th>MyMRKO Mean±SE</th>
<th>$P$</th>
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<td></td>
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<tr>
<td>Medial</td>
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<td>Cortex</td>
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<td>Primary motor</td>
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<td>Secondary motor</td>
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</table>

Values represent mean±SE of Iba1+ cells observed in different anatomic regions within the cerebrum. Cells were counted in a 40× field. N=5 to 7 per group.

Figure 4. MyMRKO shows an altered inflammatory response during MCA occlusion. mRNA expression of M1 classically activated macrophage markers (A) M2 alternatively activated macrophage markers (B) and IL-17 (C) after MCA occlusion. All genes were normalized to $\beta$-actin. n=4 per group.
inflammatory protein-1α (Figure 4A). These proinflammatory mediators are generally associated with exacerbation of tissue damage.

In contrast, the induction of M2 markers was at least partially preserved in MyMRKO. There was a significant increase in M2 markers, Arg1 and Ym1, in the ischemic hemisphere of both FC and MyMRKO groups (Figure 4B); interestingly, no significant differences in these M2 markers in the ischemic hemisphere were observed between the MyMRKO and FCs. In MyMRKO, there was a minimal suppression of Arg1 and Ym1 (less than 2-fold), whereas all of the M1 markers were suppressed by greater than 2-fold and IL-1β and monocyte chemoattractant protein-1 had a 5-fold suppression relative to controls. Other M2 markers such as the mannose receptor and Mgl1 showed no significant changes, whereas the IL-1 receptor antagonist was significantly lower in the MyMRKO group (Figure 4B).

Aldosterone has been shown to induce IL-17-mediated neuroinflammation, but we did not see any significant change in the expression of IL-17 (Figure 4C).

Because oxidative stress is a critical mediator of reperfusion injury, we determined if MyMRKO altered the expression of genes associated with oxidative stress. We found no significant stroke-induced or strain-dependent differences in several genes (reduced nicotinamide-adenine dinucleotide phosphate oxidase 2, manganese superoxide dismutase, catalase, and peroxiredoxin-2; Figure 5) that are known to contribute to oxidative damage and that are associated with inflammation. In cardiac inflammation and fibrosis, myeloid MR was shown to exacerbate tissue remodeling and increase fibrosis. However, no changes in collagen 1A, collagen 3, and fibronectin expression were detected nor did we see any difference in the expression of matrix metalloproteinase-9 (Figure 5).

**Discussion**

In the present study, we demonstrated an important role for myeloid MR during ischemic stroke using cell type-specific knockout and a model of focal cerebral ischemia. We found that deletion of MR from cells of myeloid lineage significantly reduced stroke infarct volume after MCA occlusion. Furthermore, a reduction in activated microglia/macrophages was observed along with a concomitant decrease in proinflammatory markers associated with the classically activated M1 macrophage phenotype. In addition, there was a partial preservation of the alternatively activated M2 macrophage phenotype. These data indicate that MyMRKO confers neuroprotection by modulating the immune response to ischemic stroke. Furthermore, we identify myeloid cells, which include macrophages and microglia, as critical targets for MR antagonists and MR regulation of myeloid cells as a potential mechanism for neuroprotection exhibited in previous studies.

To address the effect that myeloid MR has on the inflammatory response to stroke, we measured the expression of classical M1 and alternatively activated, M2, markers after MCA occlusion. We observed infarct-induced MyMRKO suppression of tumor necrosis factor-α, IL-1β, monocyte chemoattractant protein-1, IL-6, and macrophage inflammatory protein-1α, which are expressed principally by macrophages and are markers of classical M1 macrophage activa-
tion. Several of the M1 markers such as monocyte chemoattractant protein-1, IL-1β, and macrophage inflammatory protein-1α were highly suppressed by 5-, 5- and 3-fold changes, respectively. However, the expression of M2 markers, Arg1 and Ym1, was partially preserved in MyMRKO mice with both being suppressed by ~2-fold. The suppression of M2 markers was less than all of the M1 markers tested. This indicates there may be a higher ratio of M2 polarized myeloid cells within the brain and suggests that macrophage polarization may have an important role in neurological outcome. Other M2 markers that were previously found to be regulated by MR during cardiac inflammation such as the mannose receptor, Mgl1, and Fizz1 were not upregulated during ischemia. This is likely due to the fact that different phenotypes of M2 polarization can exist based on the external stimuli that activate macrophages or expression of these genes in other cell types.

Immunohistochemical staining for Iba1 indicates a significant change in the macrophage/microglia response. However, Iba1 does not differentiate between macrophages and microglia, and it is difficult to differentiate the 2 cell types based on morphology. Changes in Iba1 staining were mainly confined to subcortical regions, although changes in infarct size are largely defined by differences in the cortex. This could indicate that MR control of the M1/M2 phenotype, rather than increases in the total number of myeloid cells, are more important in determining infarct size in the cortex.

There is evidence that microglia also adopt different functional phenotypes similar to the classical and alternative macrophage polarization. However, microglia do not express LysM until they become activated, and even on activation, there is only partial gene recombination and deletion. Microglia containing LysM-Cre are able to undergo partial recombination during isolation and culturing, but we were unable to detect any suppression of MR expression in cultured microglia (Supplemental Figure I). This would suggest that resident macrophages or infiltrating myeloid cells might have a more dominant role in reducing inflammation and lesion size. It also remains to be determined whether MR activation can affect the population of circulating monocytes, which are recruited after ischemic stroke. Therefore, future studies aimed at identifying the individual contribution of monocytes, macrophages, and microglia are warranted.

Iwanami et al have shown that the MR antagonist eplerenone reduces macrophage-associated oxidative stress after MCA occlusion. Furthermore, high levels of aldosterone, a physiological MR ligand, increase oxidative stress in circulating monocytes as well as isolated macrophages. Our data show that myeloid MR does not affect the expression of genes associated with oxidative stress and the production of reactive oxygen species 24 hours after ischemic stroke. However, many enzymes that generate reactive oxygen species can be directly activated within minutes of ischemia and reperfusion. Furthermore, aldosterone is capable of activating reduced nicotinamide-adenine dinucleotide phosphate oxidase 2 (NOX2), manganese superoxide dismutase (MnSOD), catalase (Cat), peroxiredoxin-2 (Prdx2) nor did it show a difference in the expression of genes associated with tissue remodeling and fibrosis (collagen 1A [Col1A]).
phosphate oxidase by nongenomic mechanisms.\textsuperscript{25} We have not directly measured the levels of reactive oxygen species and therefore it could be possible that MR affects reactive oxygen species production in this manner.

Although mineralocorticoid excess clearly affects stroke, in a normal physiological setting where glucocorticoids are significantly higher than aldosterone, as mentioned, myeloid MR is thought to be mainly occupied by glucocorticoids. Glucocorticoids have been implicated as important regulators of MR function in other tissues that lack 11\beta-hydroxy-steroid dehydrogenase 2, including the brain and heart. In a model of myocardial infarction, the glucocorticoid cortisol was shown to increase the size of infarction and myocyte cell death.\textsuperscript{26} This response was blocked by the MR antagonist spironolactone indicating potential actions of glucocorticoids on MR. Therefore, in our model of ischemic stroke, it is possible that glucocorticoids have a significant role in mediating the proinflammatory effects of myeloid MR. However, it is unclear how either aldosterone or glucocorticoids alter inflammation in macrophages and other myeloid cells. Future studies need to be aimed at understanding the mechanisms of myeloid phenotypic control by MR and whether MR is a direct transcriptional activator of proinflammatory genes.

In summary, this study has identified a previously unknown role for myeloid MR activation during ischemic stroke. Using MyMRKO mice, we demonstrated that MR activation in myeloid cells exacerbates inflammation and alters the M1/M2 inflammatory response to stroke. Moreover, these experiments indicate that MR control of immune cell function significantly affects stroke lesion size.

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Disclosures
None.

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SUPPLEMENTAL MATERIAL

Methods

Measurement of Infarct Volume. Following 24 hours of reperfusion, the mice were killed and transcardially perfused with heparinized saline (1U/mL). The brains were removed and post-fixed in 4% paraformaldehyde for 1 week. The cerebrum was then cut into 1 mm thick serial coronal sections. 10 μm paraffin embedded sections were stained with hematoxylin and eosin and the cerebral infarct volume was quantified using NIH image analysis software (Image J ver 1.43). Infarct volume was corrected for brain edema.

Immunohistochemistry. Microglia and macrophage activation and localization were determined after 24 hours of reperfusion with the microglia/macrophage selective antibody ionized calcium-binding adapter protein 1 (Iba1) (Abcam) at a 1:300 dilution using standard staining protocols on paraffin embedded sections. Iba1+ cells were quantified and expressed as number of cells/field (40X objective). Two 40X fields were counted per anatomical region and averaged to obtain the number of Iba1+ microglia and macrophages.

Quantitative real-time RT-PCR. mRNA expression was measured after 24 hours reperfusion. Total RNA was extracted from frozen whole cerebral hemispheres using TRIZol reagent and then purified with the RNeasy Mini Kit (Qiagen). Purified RNA (1μg) was reverse transcribed to cDNA using an Applied Biosystems kit. QRT-PCR was performed using a Bio-Rad iCycler. The relative mRNA expression was quantified using the comparative method and mRNA was normalized to β-actin.

Laser Doppler Flowmetry and Blood Gas. Cortical perfusion in the MCA territory was measured used laser Doppler flowmetry and was determined before and during occlusion of the
MCA. For measurement of pH, PO₂, and PCO₂, a catheter was implanted into the femoral artery and arterial blood was collected during pre-ischemic and ischemic periods.

**Microglia Isolation and Culture.** Isolation of cerebral microglia was performed as described elsewhere¹. Briefly, 10-12 wk old mice FC and MyMRKO mice were euthanized and transcardially perfused with heparinized saline (1U/mL). The cerebrum was homogenized in ice cold PBS in a Tenbroeck homogenizer. The homogenate was then filtered through a 50 μm strainer and then resuspended in 70 % isotonic Percoll. A 0/40/70% Percoll gradient was set up and centrifuged at 1200 x g for 45 min at 20°C. The microglia containing fraction was then collected, resuspended in RPMI + 10% FBS and plated at a density of 2 x 10⁵ cells/mL/well. Cells were washed with PBS (+ calcium chloride, + magnesium chloride) after 2 hours to remove non-adherent cells and then incubated for 24 hours at 37 °C, 5% CO₂.
Figure Legend

S2. Expression of MR in cultured microglia. Microglia were isolated from the cerebrum of FC and MyMRKO mice and cultured for 24 hours. No significant change in MR expression was detected between FC and MyMRKO mice. N = 5 per group.
Table S1. Cerebral blood flow and arterial blood gas measurements.

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Values represent mean ± S.E. The ischemic cerebral blood flow (CBF) is represented as the percentage of the pre-ischemic, baseline CBF. There were no significant differences between FC and MyMRKO mice (N = 4 per group). FC = Floxed Control, MyMRKO = myeloid MR knockout.
Figure S2.
Supplemental References