Mineralocorticoid Receptor Deficiency in Macrophages Inhibits Neointimal Hyperplasia and Suppresses Macrophage Inflammation Through SGK1-AP1/NF-κB Pathways


Objective—Restenosis after percutaneous coronary intervention remains to be a serious medical problem. Although mineralocorticoid receptor (MR) has been implicated as a potential target for treating restenosis, the cellular and molecular mechanisms are largely unknown. This study aims to explore the functions of macrophage MR in neointimal hyperplasia and to delineate the molecular mechanisms.

Approach and Results—Myeloid MR knockout (MMRKO) mice and controls were subjected to femoral artery injury. MMRKO reduced intima area and intima/media ratio, Ki67- and BrdU-positive vascular smooth muscle cells, expression of proinflammatory molecules, and macrophage accumulation in injured arteries. MMRKO macrophages migrated less in culture. MMRKO decreased Ki67- and BrdU-positive macrophages in injured arteries. MMRKO macrophages were less Ki67-positive in culture. Conditioned media from MMRKO macrophages induced less migration, Ki67 positivity, and proinflammatory gene expression of vascular smooth muscle cells. After lipopolysaccharide treatment, MMRKO macrophages had decreased p-cFos and p-cJun compared with control macrophages, suggesting suppressed activation of activator protein-1 (AP1). Nuclear factor-κB (NF-κB) pathway was also inhibited by MMRKO, manifested by decreased p-1κB kinase-β and p-1κBα, increased 1κBα expression, decreased nuclear translocation of p65 and p50, as well as decreased phosphorylation and expression of p65. Finally, overexpression of serum-and-glucocorticoid-inducible-kinase-1 (SGK1) attenuated the effects of MR deficiency in macrophages.


Key Words: femoral artery ▪ hyperplasia ▪ macrophage ▪ mineralocorticoid receptor ▪ myeloid cells

Restenosis after percutaneous coronary intervention (PCI) is still an important clinical problem that is far from being solved. The rate of restenosis has remained >10% even after the introduction of drug-eluting stents aiming to suppress neointimal proliferation.1 Furthermore, it is particularly challenging to treat restenosis after placement of drug-eluting stents currently available.2 New strategies have been developed and new targets have been identified with the expectation to achieve better outcome for treating restenosis. Although experimental data have identified mineralocorticoid receptor (MR) as a potential target for treating restenosis after PCI, clinical evidence is controversial. Eplerenone, an antagonist of MR, suppresses vascular remodeling after...
Nonstandard Abbreviations and Acronyms

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<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>AP1</td>
<td>activator protein 1</td>
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<td>CM</td>
<td>conditioned media</td>
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<td>IL</td>
<td>interleukin</td>
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<td>LC</td>
<td>littermate control of MMRKO</td>
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<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
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<td>MCP1</td>
<td>monocyte chemotactic protein-1</td>
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<td>MMRKO</td>
<td>myeloid MR knockout</td>
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<td>MR</td>
<td>mineralocorticoid receptor</td>
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<td>NF-κB</td>
<td>nuclear factor κB</td>
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<td>PCI</td>
<td>percutaneous coronary intervention</td>
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<td>SMA</td>
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<td>SGK1</td>
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angioplasty of coronary arteries and neointima formation after stent implantation in coronary arteries of swine. However, the effects of MR antagonists on restenosis in human patients are uncertain. Early study has shown that spironolactone, another antagonist of MR, does not change the incidence of restenosis at 6 months after stenting in a clinical trial. More recent results, however, have demonstrated that spironolactone reduces the rate of repeat revascularization at 1 year after PCI. Different population, length of treatments, and end points between the 2 studies may have contributed to the differential results. More fundamental studies on the cellular and molecular mechanisms how MR is involved in restenosis are needed to provide new insights for ultimately using this target to treat restenosis.

Recent studies using knockout mouse models have begun to reveal the cell type–specific influence of MR in the vasculature. MR in monocytes/macrophages may play important roles in neointimal hyperplasia. Myeloid MR knockout (MMRKO) mouse was established and used to study the functions of macrophage MR in the cardiovascular system. The data have illustrated the importance of macrophage MR in cardiac hypertrophy, fibrosis, and inflammation. Much less has been explored in the vasculature using MMRKO mice. Decreased vascular fibrosis and remodeling have been observed in MMRKO mice challenged with Nω-nitro-ω-arginine methyl ester/angiotensin II or pressure overload. However, these 2 hypertensive models mostly induce remodeling in the adventitia of blood vessels without neointimal formation, and the pathological changes are distinctly different from those during restenosis. Therefore, it has remained unexplored whether macrophage MR is critical in the process of neointimal hyperplasia. Moreover, although MR has been shown to control macrophage polarization and inflammation, little is known about the molecular mechanisms.

In this study, we aim to explore the functions of macrophage MR in neointimal hyperplasia as well as the cellular and molecular mechanisms. We first use a classical femoral wire injury mouse model that resembles the pathological changes after PCI to investigate whether MR deficiency in myeloid cells reduces neointimal hyperplasia and vascular inflammation. Then, we study the cellular mechanisms by evaluating the impacts of MR deletion on migration and proliferation of macrophages, as well as the interactions between macrophages and vascular smooth muscle cells (VSMCs). Finally, we further explore the intrinsic molecular signaling pathways that mediate the effects of MR deletion in inflammatory response of macrophages.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Myeloid MR Deficiency Inhibits Arterial Injury–Induced Neointimal Hyperplasia

After wire injury, femoral arteries of MMRKO mice had significantly reduced intima area, intima/media ratio, and thickness of neointima compared with littermate control (LC) mice (Figure 1A and 1B; Figure 1A in the online-only Data Supplement). No difference was detected in the sham-operated arteries between LC and MMRKO mice (Figure 1A and 1B). Next, we evaluated proliferation of VSMCs by immunofluorescence staining of α-smooth muscle actin (SMA) and Ki67. SMA-positive cells were significantly less in the injured femoral arteries of MMRKO mice (Figure 1C and 1D). Furthermore, SMA and Ki67 double-positive cells were much less in MMRKO mice, indicating reduced proliferation of VSMCs (Figure 1C and 1E). Consistently, SMA and BrdU double-positive cells were significantly less in the injured arteries of MMRKO mice (Figure 1F and 1G). In addition, MMRKO mice showed less vascular fibrosis measured by picrosirius red staining (Figure 1H and 1I). In sham-operated mice, SMA-positive cells were comparable between LC and MMRKO, and Ki67 was not detected in either genotype (Figure 1B and 1C in the online-only Data Supplement). Staining of CD31 demonstrated similar endothelial coverage between LC and MMRKO mice (Figure 1D in the online-only Data Supplement).

Myeloid MR Deficiency Inhibits Arterial Injury–Induced Vascular Inflammation

Quantitative reverse transcription polymerase chain reaction results showed that injured arteries of MMRKO mice had much lower expression of proinflammatory genes, such as interleukin-6 (IL-6), IL-1β, intercellular adhesion molecule-1, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, macrophage inflammatory protein-2α, inducible nitric oxide synthase, and matrix metalloproteinase-9 (Figure 2A). The 2 groups had comparable expression of other genes, including tumor necrosis factor-α, chemokine (C–C motif) ligand 1, regulated on activation, normal T-cell expressed and secreted, and cyclooxygenase 2 (Figure 2A). Immunohistochemical staining showed markedly attenuated staining of IL-6 and IL-1β in MMRKO mice (Figure 2B and 2C).

We then examined accumulation of macrophages within femoral arteries by F4/80 and Mac2 immunohistochemical
staining. Injured arteries from MMRKO mice exhibited decreased expression of F4/80 and Mac2 compared with those from LC mice (Figure 2B and 2C). Consistently, injured arteries from MMRKO mice had significantly less gene expression of F4/80 and Mac2 (Figure 2D).

Relative amounts of total Ly6Chigh and Ly6Clow monocytes in the blood were comparable between LC and MMRKO mice after wire injury (Figure III in the online-only Data Supplement).

**Myeloid MR Deficiency Inhibits Macrophage Migration**

The diminished macrophage accumulation in the vascular wall of MMRKO mice responding to wire injury could be attributable to at least 2 events, less migration of monocytes and less proliferation of macrophages. We first investigated the possibility of reduced migration of monocytes/macrophages.
Figure 2. Myeloid mineralocorticoid receptor deficiency inhibits arterial injury-induced vascular inflammation. A, Expression of proinflammatory genes in femoral arteries from littermate control (LC) or knockout (KO) mice 7 days after sham operation or wire injury (injured). Ten mice were used for each group and samples from every 3 to 4 mice were pooled. B, Representative immunohistochemical staining of interleukin (IL)-6, IL-1β, F4/80, and Mac2 in femoral arteries 21 days after wire injury (n=5). Positive cells stained brown. Scale bar, 200 μm. C, Quantification of IL-6, IL-1β, F4/80, and Mac2-positive areas. D, Gene expression of macrophage markers in femoral arteries 7 days after sham operation or wire injury. All gene expression was measured using quantitative reverse transcription polymerase chain reaction. COX2 indicates cyclooxygenase 2; CXCL1, chemokine (C-X-C motif) ligand 1; ICAM1, intercellular adhesion molecule-1; iNOS, inducible nitric oxide synthase; MIP, macrophage inflammatory protein; MMP9, matrix metalloproteinase-9; ns, not significant; RANTES, regulated on activation, normal T-cell expressed; and TNFα, tumor necrosis factor-α.
Migration of monocytes/macrophages is determined by 2 aspects, the amount of stimulating factors and the migratory ability of monocytes/macrophages responding to these factors. Cytokines such as monocyte chemoattractant protein-1 (MCP1) and osteopontin are known to play essential roles in macrophage migration.12–14 Gene expression of MCP1 and its receptors, chemokine (C–C motif) receptor 2 and chemokine (C–C motif) receptor 4, as well as osteopontin was much lower in the injured arteries of MMRKO mice (Figure 3A). Immunohistochemical staining of MCP1 and osteopontin showed that protein levels of these cytokines were also markedly reduced (Figure 3B and 3C).

Transwell assay displayed that the migratory ability of peritoneal macrophages from MMRKO mice was markedly inhibited both at baseline and when stimulated by either MCP1 (Figure 3D and 3E) or osteopontin (Figure 3F and 3G). Gene expression of MCP1 and osteopontin in peritoneal macrophages showed no difference between LC and MMRKO (Figure IV in the online-only Data Supplement), suggesting that the difference observed in femoral arteries was not from macrophages. However, gene expression of chemokine (C–C motif) receptor 2 and chemokine (C–C motif) receptor 4 was significantly suppressed in MMRKO macrophages (Figure 3H), further supporting the impaired migratory ability of these macrophages.

Taken together, MMRKO decreased migration of monocytes/macrophages probably by reducing the amount of cytokines in the vascular wall after injury and suppressing the migratory ability of macrophages.

**Myeloid MR Deficiency Inhibits Macrophage Proliferation**

We then addressed the second possibility that MMRKO decreased proliferation of macrophages. Mac2-positive cells were significantly decreased in the injured arteries of MMRKO mice compared with LC mice (Figure 4A and 4B). Interestingly, Mac2 and Ki67 double-positive cells were readily detected in the injured arteries, indicating the existence of proliferative macrophages in this model (Figure 4A and 4C). Furthermore, the double-positive cells were much less in MMRKO mice, suggesting reduced proliferation of macrophages (Figure 4A and 4C). Similarly, Mac2 and BrdU double-positive cells were significantly less in the injured arteries of MMRKO mice (Figure 4D and 4E). Similar results were obtained when CD68 was used to detect macrophages (Figure V in the online-only Data Supplement). In sham-operated mice, neither Mac2 nor Ki67 was detected in LC or MMRKO group (Figure VI in the online-only Data Supplement).

Proliferation of macrophages is also determined by 2 aspects, the amount of stimulating factors and the proliferative ability of macrophages responding to these factors. Macrophage colony-stimulating factor (M-CSF) and IL-4 are the 2 important factors that promote macrophage proliferation.15,16 Interestingly, gene expression of M-CSF, but not IL-4, was markedly lower in the injured arteries of MMRKO mice (Figure 4F; Figure VIIA in the online-only Data Supplement).

Peritoneal macrophages from MMRKO mice had decreased gene expression of CSF-1R, the receptor of M-CSF (Figure 4G), implicating reduced proliferative ability of MR-deficient macrophages. We then assessed proliferation of macrophages responding to L929-conditioned media (CM) in which M-CSF is the major stimulator. Mac2 and Ki67 staining showed that peritoneal macrophages from MMRKO mice had significantly less proliferation with or without L929 CM stimulation (Figure 4H and 4I). Consistently, MMRKO macrophages had decreased protein expression of proliferating cell nuclear antigen and increased protein expression of P27 (Figure 4J and 4K). Gene expression of M-CSF in peritoneal macrophages showed no difference between LC and MMRKO (Figure VIIB in the online-only Data Supplement), suggesting that the difference observed in femoral arteries was not from macrophages. These data together demonstrated that MMRKO inhibited macrophage proliferation probably through reducing the availability of M-CSF in the microenvironment of injured arteries and downregulating the proliferative ability of macrophages.

**Conditioned Media From MR-Deficient Macrophages Induce Less Migration, Proliferation, and Proinflammatory Gene Expression of VSMCs**

VSMC migration, proliferation, and cytokine production are crucial pathophysiological processes in restenosis.17 CM were collected from LC or MRKO macrophages treated without lipopolysaccharide (LPS; designated as CM) or with LPS (designated as LPS CM) and used to stimulate mouse primary VSMCs. Transwell assay showed that LPS CM derived from MRKO macrophages induced markedly less migration of VSMCs than those from LC macrophages (Figure 5A and 5B). Similarly, much less Ki67-positive VSMCs were observed in the group treated with LPS CM derived from MRKO macrophages (Figure 5C and 5D). In addition, VSMCs treated with LPS CM from MRKO macrophages had much lower expression of proinflammatory genes, such as IL-6, IL-1β, regulated on activation, normal T-cell expressed and secreted, and vascular cell adhesion molecule-1 (Figure 5E). Expression of other proinflammatory genes was not significantly different between the 2 groups (Figure VIII A in the online-only Data Supplement).

The concentrations of LPS in LPS CM from LC and MRKO macrophages were similar (Figure VIII B in the online-only Data Supplement), suggesting that the different impacts on VSMCs were not likely because of LPS. Instead, the difference may be attributable to the much lower levels of IL-6 and IL-1β in LPS CM from MRKO macrophages (Figure VIIIC in the online-only Data Supplement). Gene expression of IL-6 and IL-1β was among the most dramatically increased responding to LPS and suppressed in MRKO macrophages (Figure VIID in the online-only Data Supplement). When neutralizing antibody against IL-6 and that against IL-1β were added together, the different impacts of LPS CM derived from LC and MRKO macrophages on migration, proliferation, and inflammatory gene expression of VSMCs were abolished, further supporting that IL-6 and IL-1β mediated the effects of MRKO (Figure 5A–5E).

**MR Deficiency Impairs AP1 and NF-κB Signaling Pathways in Macrophages**

We next explored whether MRKO affected the intrinsic pathways that control the release of proinflammatory cytokines,
such as IL-6 and IL-1β in macrophages. LC or MRKO macrophages were treated with LPS and activation of activator protein 1 (AP1), and nuclear factor-κB (NF-κB) pathways were detected. LPS-induced phosphorylation of both c-Fos and c-Jun, components of AP1, was significantly lower in peritoneal macrophages from MMRKO mice compared with those
Figure 4. Myeloid mineralocorticoid receptor deficiency inhibits macrophage proliferation. 

A, Representative immunofluorescence staining of macrophages in injured arteries from littermate control (LC) or knockout (KO) mice. Yellow arrows indicate Mac2-positive macrophages and white arrows indicate Mac2 and Ki67 double-positive cells. 

B, Quantification of Mac2-positive macrophages. 

C, Quantification of Mac2 and Ki67 double-positive cells. Bonferroni post-tests: *P<0.05, **P<0.01 versus KO. 

D, Representative immunofluorescence staining of Mac2 and BrdU in femoral arteries 21 days after wire injury (n=4). The arrows point to Mac2/BrdU double-positive cells. 

E, Quantification of Mac2 and BrdU double-positive cells. 

F, Gene expression of macrophage colony-stimulating factor (M-CSF) in femoral arteries 7 days after sham operation or wire injury. 

G, Gene expression of CSF-1R in peritoneal macrophages (n=6). 

H, Representative immunofluorescence staining of peritoneal macrophages treated with or without L929-conditioned media (CM) for 48 hours. 

I, Quantification of Ki67-positive macrophages. 

J, Western blotting analysis of proliferating cell nuclear antigen (PCNA) and p27 of peritoneal macrophages. 

K, Quantification of J. All gene expression was measured using quantitative reverse transcription polymerase chain reaction. All scale bars: 200 μm. External elastic lamina in all magnified images was marked by yellow-dashed lines. ns: not significant.
from LC mice (Figure 6A–6C). These results suggested that MRKO suppressed AP1 activation in macrophages.

The upstream IκB kinase β was much less phosphorylated in MRKO macrophages after LPS treatment (Figure 6D and 6E). Consistently, phosphorylation of IκBα was suppressed in MRKO macrophages while total protein level of IκBα was increased in comparison with LC macrophages (Figure 6D and 6F). Furthermore, markedly less nuclear p65-positive cells were observed in MRKO macrophages after LPS stimulation (Figure 6G and 6H). Similarly, MRKO also inhibited nuclear translocation of NF-κB p50 (Figure IX in the online-only Data Supplement). In addition, both p-p65 and total p65 were attenuated in MRKO macrophages (Figure 6I, 6J, and 6K). These results together suggested that MRKO inhibited NF-κB activation in macrophages.

**MR Deficiency Suppresses Inflammatory Response and AP1 and NF-κB Signaling Pathways via SGK1 in Macrophages**

Previous studies have demonstrated that serum-and-glucocorticoid-inducible-kinase (SGK1), a classical target gene of MR, plays crucial roles in vascular remodeling and regulates inflammatory response in monocytes/macrophages via NF-κB.18–20 Our results demonstrated that SGK1 deficiency largely recapitulated the phenotypes of MRKO in macrophages (Figure X in the online-only Data Supplement).

We therefore hypothesized that SGK1 mediated the effects of MRKO. MRKO markedly reduced gene expression, protein expression, and phosphorylation of SGK1 (Figure 7A–7C). Next, we addressed whether SGK1 mediated the effects of MR using Raw264.7 cells with knockdown of MR and overexpression of constitutively active mutant SGK1 (S422D, SGK1-OV; Figure XI in the online-only Data Supplement). MR siRNA markedly suppressed expression of proinflammatory genes such as IL-6 and IL-1β in Raw264.7 cells treated with LPS and SGK1-OV noticeably blunted such suppression (Figure 7D). Furthermore, the suppressive effects of MR siRNA on AP1 signaling was attenuated by SGK1-OV (Figure 7E and 7F). Similarly, MR siRNA suppressed NF-κB signaling and such effects were either blocked or inhibited by SGK1-OV (Figure 7G–7J). SGK1-OV alone had the opposite effect.
MR is an important drug target in the cardiovascular system. Its antagonists (spironolactone and eplerenone) are part of the standard therapeutic regimen for treating heart failure in clinical practice. Although animal studies have shown promising data that these antagonists may be beneficial for restenosis after PCI, they have not been clinically proved. Using a femoral artery wire injury model, in which the endothelial layer is severely damaged along with medial injury by guest on July 9, 2017 http://atvb.ahajournals.org/ Downloaded from
and intense mechanical stretching, we demonstrated that MR deficiency in macrophages effectively suppressed neointimal hyperplasia, vascular inflammation, migration and proliferation of monocytes/macrophages. Under inflammatory stimulation, MR-deficient macrophages produced less IL-1β and IL-6 and caused less activation of VSMCs. Intrinsically, MR deficiency led to downregulation of AP1 and NF-κB pathways dependent of SGK1.

We have previously demonstrated the protective roles of macrophage MR deficiency in hypertensive vascular remodeling.9,11 This study revealed that blockade of MR in macrophages was sufficient to attenuate wire injury–induced neointimal hyperplasia effectively. This indicates that macrophage MR plays an essential role in the process of restenosis after PCI. It is conceivable to treat restenosis by selectively blockade of MR in monocytes/macrofages using delivery vehicles, such as liposomes or β,1,3-glucan.23,24 These approaches may decrease the side effects of MR blockers, and high dosage becomes feasible for targeting monocytes/macrophages specifically.

MR deficiency affected macrophages in multiple ways. First, we discovered that macrophage proliferation occurred within the vascular wall in the process of neointimal hyperplasia and that MRKO dramatically suppressed the proliferation. The origin of macrophages in the context of inflammation has previously been attributed to recruitment

Figure 7. Mineralocorticoid receptor (MR) deficiency inhibits inflammatory response and activator protein-1 (AP1) and nuclear factor-κB signaling pathways via serum-and-glucocorticoid-inducible-kinase (SGK1) in macrophages. A, Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of SGK1 gene expression in littermate control (LC) and MR knockout (MRKO) peritoneal macrophages. B, Western blotting analysis of SGK1 phosphorylation and expression in LC and MRKO peritoneal macrophages. C, Quantifications of B, D, qRT-PCR analysis of proinflammatory genes in Raw264.7 cells with stable overexpression of SGK1 (SGK1-OV) or enhanced green fluorescent protein (EGFP) and transfected with MR siRNA or negative control siRNA (siRNA). All 4 groups were treated with lipopolysaccharide (LPS; 100 ng/mL) for 2 hours. E, Western blotting analysis of phosphorilation and expression of c-Fos and c-Jun in the Raw264.7 cells treated with LPS for 30 minutes. F, Quantifications of E, G, Western blotting analysis of IκB kinase β (IκKβ) phosphorylation and expression in the Raw264.7 cells treated with LPS for 15 minutes. H, Quantification of G, I, Western blotting analysis of phosphorylation and expression of IκBα and p65 in the Raw264.7 cells treated with LPS for 30 minutes. J, Quantifications of I.
of blood monocytes. However, recent studies have indicated that macrophage proliferation also plays critical roles under inflammatory conditions, such as atherosclerosis and obesity. We used 2 different approaches to show the presence of proliferative macrophages in wire-injured blood vessels. Furthermore, MRKO inhibited the proliferation of macrophages both in vivo and in vitro. Second, MRKO markedly suppressed the migration of monocytes/macrophages. The reduced macrophage accumulation in the vascular wall of MMKO mice after wire injury is likely attributable to both decreased migration of monocytes and attenuated proliferation of macrophages. However, which event contributes more significantly and whether the proliferated macrophages are initially originated from circulating monocytes remains to be further interrogated. Third, MRKO macrophages produced less proinflammatory cytokines such as IL-1β and IL-6 that, in turn, mediated the activation of VSMCs. Earlier study has shown that conditioned media from aldosterone-stimulated VSMCs promote monocyte chemotaxis in an MR-dependent manner. Therefore, MR mediates the crosstalk between macrophages and VSMCs.

We identified SGK1-AP1/NF-κB pathways as the underlying molecular mechanisms through which MR regulates the inflammatory response of macrophages. Previously, spironolactone has been reported to decrease the DNA-binding activity of AP1 and NF-κB, as well as gene expression of c-Fos in cardiac tissues of renin and angiotensinogen double transgenic rats that overproduce angiotensin II. In spontaneously hypertensive rats, eplerenone decreases aortic expression of IL-1β, IL-6, and tumor necrosis factor-α as well as plasma levels of IL-1β and IL-6, likely through upregulation of 1xκB expression and downregulation of NF-κB expression. In renal collecting duct cells, aldosterone works through an MR-SGK1/NF-κB pathway to promote expression of NF-κB target genes, including IL-1β and IL-6. Similar pathway has been reported in mesangial cells. However, the molecular mechanisms how MR regulates inflammation in macrophages had been little explored. Our data specifically showed that MR deficiency in macrophages diminished activation of both AP1 and NF-κB in these cells and these effects were dependent on SGK1. A recent study has demonstrated that SGK1 deficiency suppresses NF-κB through inhibition of p-IκK, p-IκB, and P50 nuclear translocation in macrophages and that constitutively active SGK1 conversely activates NF-κB. Our results further demonstrated that SGK1 deficiency in macrophages suppressed LPS-induced proinflammatory gene expression and that both AP1 and NF-κB pathways were downregulated. Therefore, MR deficiency suppresses inflammation in macrophages by downregulation of SGK1, which in turn controls the activation of AP1 and NF-κB that both govern the expression of inflammatory genes, such as IL-1β and IL-6.

Together our data have revealed MR in macrophages as a critical player in neointimal hyperplasia in response to vascular injury and have delineated the intrinsic mechanisms how MR regulates inflammatory response in macrophages. These data have provided new insights on how to target monocytes/macrophages to curb inflammation that is the propelling engine of neointimal hyperplasia in the process of restenosis.

Sources of Funding
This work was supported by grants from the Ministry of Science and Technology of China (973 Program 2012CB524900), the National Natural Science Foundation of China (91339110, 31371153, 31171133, 31400987), Science and Technology Commission of Shanghai Municipality (15140904400), and Key Laboratory of Nutrition and Metabolism, Chinese Academy of Sciences (KLM201401). S.Z. Duan was supported by the 100 Talents Program of the Chinese Academy of Sciences (2012H0TP06).

Disclosures
None.

References


**Significance**

Mineralocorticoid receptor (MR), an important drug target for the cardiovascular system, has been implicated to be useful for treating restenosis after percutaneous coronary intervention. However, the cellular and molecular mechanisms are largely unknown. This study first demonstrated the important functions of macrophage MR by showing that MR deficiency in macrophages effectively suppressed neointimal hyperplasia, vascular inflammation, macrophage proliferation, and migration, as well as activation of vascular smooth muscle cells. The study further identified MR-serum-and-glucocorticoid-inducible-kinase-1-activator protein-1/nuclear factor-xB signaling pathways as the underlying molecular mechanisms through which MR regulates the inflammatory response of macrophages. These results support macrophage MR and serum-and-glucocorticoid-inducible-kinase-1-activator protein-1/nuclear factor-xB axis as important targets for blocking neointimal hyperplasia and potentially treating restenosis.
Mineralocorticoid Receptor Deficiency in Macrophages Inhibits Neointimal Hyperplasia and Suppresses Macrophage Inflammation Through SGK1-AP1/NF-κB Pathways
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Arterioscler Thromb Vasc Biol. 2016;36:874-885; originally published online March 10, 2016; doi: 10.1161/ATVBAHA.115.307031
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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MATERIALS AND METHODS

Animals and Treatment
Myeloid MR knockout (MRKO) and littermate control (LC) mice were generated as before. Myeloid SGK1 knockout (SGK1-KO) and their littermate control (SGK1-LC) mice were obtained by crossing floxed SGK1 mice with LysM-Cre mice. Bromodeoxyuridine (BrdU, 25mg/kg, Sigma-Aldrich, St. Louis, MO) was administrated to mice via subcutaneous injection every day for 3 weeks after surgeries. All animal studies were approved by the Institutional Animal Care and Use Committee of Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Femoral Artery Wire Injury Model
Twelve-week-old male MRKO or LC mice were subjected to wire injury of femoral artery as previously describe. Mice were anesthetized with isoflurane and right femoral arteries were exposed by blunt dissection. A guide wire 0.38 mm in diameter (Cook Inc, Bloomington, IN) was inserted into the arterial lumen and advanced to the level of the aortic bifurcation and then pulled back. The process was repeated for two more times and the guide wire was left in place for 2 minutes to denude the endothelium. Sham surgeries were performed on the left femoral arteries of the same mice. Mice were sacrificed at indicated time points after injury and the femoral arteries were collected for histologic or biochemical analysis.

Histology and Morphometric Analysis
Femoral arteries were perfused and fixed with 4% paraformaldehyde and embedded in paraffin. Serial cross-sections (5μm) were cut and stained with hematoxylin and eosin. Intimal area, medial area, and total vascular area were measured using Image J software (National Institutes of Health, Bethesda, MD). The intima to media ratio was calculated. Sections were stained with 0.1% picrosirius red and fibrosis was quantified as the ratio of the positively stained area to the total area using Image-Pro Plus software (Media Cybernetics, Rockville, MD).

Immunofluorescence and Immunohistochemistry
For immunofluorescence, paraffin sections were deparaffinized and rehydrated. After antigen retrieval treatment the slides were sequentially incubated in blocking buffer at room temperature for 60 minutes, with primary antibodies at 4°C overnight, and then with fluorochrome-conjugated secondary antibody (1:1000, Life Technologies, Grand Island, NY) at 37°C for 45 minutes. Nuclei were stained by DAPI contained in mounting media (Life Technologies). For immunocytochemistry, cells were cultured on coverslips and treated. At the end of experiments, the coverslips were fixed with 4% formaldehyde at room temperature for 15 minutes. After being blocked in PBS containing 5% normal goat serum and 0.3% Triton X-100 (Sigma-Aldrich) for 1 hour, cells were incubated with primary antibodies at 4°C overnight, and then stained with secondary antibody at 37°C for 45 minutes. The fluorescent signals were captured and images were acquired using a Zeiss confocal microscope (Carl Zeiss, Oberkochen, Germany).

For immunohistochemistry, paraffin sections were first incubated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase. Slides were then incubated with primary antibodies at 37°C for 60 minutes, followed by incubation with horseradish peroxidase-conjugated secondary antibody and DAB for color development. The staining was quantified using Image Pro Plus software and expressed as percentages of positively stained areas to total areas.

Primary antibodies for immunofluorescence and immunocytochemistry were used at the following dilutions: anti-α-smooth muscle actin (1:100, Abcam, Cambridge, MA), anti-Ki67 (1:200, Cell Signaling Technology, Danvers, MA), anti-CD31 (1:100, BD
Biosciences, San Jose, CA), anti-BrdU (1:200, Santa Cruz Biotechnology, Dallas, TX), anti-Mac2 (1:200, eBioscience, San Diego, CA), anti-CD68 (1:50, AbD Serotec, Kidlington, UK), anti-p65 and p50 (1:50, Santa Cruz Biotechnology, Dallas, TX). Primary antibodies were replaced by mouse or rat IgG (1:100, Cell Signaling Technology) for negative controls (Figure IE in the online-only Data Supplement).

Primary antibodies for immunohistochemistry were used at the following dilutions: anti-IL-6 (1:200, Abcam), anti-IL-1β (1:200, Abcam), anti-Mac2 (1:200, eBioscience), anti-F4/80 (1:50, Abcam), anti-MCP1 (1:50, Abcam), and anti-OPN (1:50, Abcam). Primary antibodies were replaced by rabbit IgG (1:100, Cell Signaling Technology) for negative control (Figure II in the online-only Data Supplement).

Flow cytometry analysis
Anticoagulated peripheral blood was collected from mice and red blood cells were removed using lysis buffer. White blood cells (1x10^6) were incubated with Fc receptor antibody (BD Biosciences) on ice for 15 minutes to reduce non-specific binding, and then incubated with FITC-conjugated CD11b (1:200, eBioscience), APC-conjugated Ly6G (1:200, BD Biosciences), PE-conjugated Ly6C (1:200, BD Biosciences) on ice for 40 minutes. The cells were then analyzed using flow cytometry (FACS Aria; BD Biosciences). Monocytes were identified as CD11b^high/Ly6G^low and neutrophils as CD11b^high/Ly6G^high.

Cell Culture
Peritoneal macrophages were obtained as previously described. Primary VSMCs from aorta of wild type C57 mice were cultured as previously described. VSMCs from passage 3 to 5 were used. L929 cells were cultured using RIPA1640 (Life Technologies) containing 10% inactivated FBS (Life Technologies) for 7 days and then L929 CM were collected.

Macrophages were treated with or without lipopolysaccharide (LPS, 100ng/ml, Sigma-Aldrich) for 24 hours and conditioned media (CM) were collected for VSMC co-culture and ELISA assay. For neutralization, LPS CM derived from peritoneal macrophages were incubated with mouse neutralizing antibody against IL-6 and that against IL-1β (both 1:400, Abcam) at 4°C overnight before being used for VSMC co-culture.

Cell Migration Assay
Cell migration was assayed using transwell plates (polycarbonate membranes with 8 µm pores, Corning Inc., Corning, NY). Briefly, peritoneal macrophages (5x10^5) or primary VSMCs (1x10^5) were seeded into the inner chamber and cultured with serum-free medium. For macrophage migration, complete medium containing recombinant murine MCP1 (20ng/ml, Sigma-Aldrich) or recombinant murine OPN (20µg/ml, Sigma-Aldrich) were added to the outer chamber. Cells were allowed to migrate at 37°C in a humidified CO2 incubator for 24 hours. Complete medium without chemokines were used as control. For VSMC migration, macrophage CM with or without LPS was added to the outer chamber and the cells were allowed to migrate at 37°C for 6 hours. All cells were fixed with methanol for 5 minutes and stained with 0.1% crystal violet (Sigma-Aldrich) for 20 minutes. Experiments were repeated 3 times.

Quantitative RT-PCR
Total RNA from femoral arteries or cells was isolated using Trizol (Life Technologies) and cDNA was synthesized using reverse transcription kits (Clontech Laboratories, Inc., Mountain View, CA) according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green Mix (Life Technologies) on an iCycler (Bio-Rad, Hercules, CA). Primer sequences were listed (Table I in the
online-only Data Supplement).

Western Blotting

Total protein was extracted by RIPA buffer containing protease inhibitors according to the manufacturer’s protocol (Beyotime Biotechnology, Shanghai, China) and quantified using BCA Protein Assay Kit (Pierce, Rockford, IL). Protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat milk in triethanolamine-buffered saline (TBS) with 0.1% Tween-20 at room temperature for 1 hour and then incubated with primary antibodies at 4°C overnight, followed by detection with horseradish peroxidase-conjugated secondary antibodies (1:1000, Proteintech, Chicago, IL). An ECL detection kit (Life Technologies) was used to detect protein expression levels. The western blotting results were quantified using Image J software.

Primary antibodies were used at the following dilutions: PCNA (1:2000, Abcam), P27 (1:1000, Abcam), β-actin (1:40000, Cell Signaling Technology), GAPDH (1:40000, Cell Signaling Technology), p-cFos and t-cFos (1:1000, Cell Signaling Technology), p-cJun and t-cJun (1:1000, Cell Signaling Technology), p-IKKβ and t-IKKβ (1:1000, Cell Signaling Technology), p-IkBα and t-IkBα (1:1000, Cell Signaling Technology), p-p65 (1:1000, Cell Signaling Technology), p65 (1:1000, Santa Cruz Biotechnology), p-SGK1 (1:1000, Cell Signaling Technology), SGK1 (1:1000, Abcam), p-NDRG1 (1:1000, Abcam), HA-Tag (1:1000, Cell Signaling Technology).

ELISA Assay

Cell culture supernatants were collected from macrophages CM with or without LPS stimulation. IL-6 and IL-1β were measured using mouse ELISA kits (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions.

Measurement of LPS Concentration

LPS concentration of macrophage CM was detected by Limulus amebocyte lysate (LAL) assay. Experiments were carried out according to the protocol from ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ).

Generation of Raw264.7 Cell Line with SGK1 Overexpression (SGK1-OV)

Constitutively active mutant rat HA-SGK1 (S422D) was subcloned into pHAGE-fEF1a-IREZ-ZsGreen plasmid and then introduced into 293FT cells together with lentivirus packaging plasmids using Lipofectamine 2000 (Life Technologies). Media containing lentivirus were harvested 48 hours after transfection, filtered through 0.22 μm filters, and used to infect RAW264.7 cells. After 3-4 time passages, RAW264.7 cells with stable overexpression of HA-SGK1 (S422D) were selected by two rounds of GFP sorting using flow cytometry.

MR RNA Interference in Raw264.7 Cell Lines

RNA interference in Raw264.7 cells was carried out by siRNA transfection reagent (Santa Cruz Biotechnology) according to manufacturer’s instructions. After 48 hours of RNA interference, cells were treated with LPS and collected for QRT-PCR and western blotting. Mouse MR siRNA sequence and negative control (scrambled) siRNA were purchased from Santa Cruz Biotechnology (sc-38837 and sc-37007) and a concentration of 40pmol/ml was used.

Statistical Analysis

Data were presented as mean ± SEM. Data analysis was performed by Prism 5.0 (GraphPad Software, La Jolla, CA). Pair-wise comparisons were analyzed by Student’s t test. Multiple comparisons were assessed by a 2-way ANOVA and Bonferroni
post-tests. Values of $p \leq 0.05$ were considered statistically significant.

References


### SUPPLEMENTAL MATERIAL

#### Supplemental Table I. List of mouse primers for QRT-PCR.

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Supplemental Figure I. Myeloid MR deficiency inhibits arterial injury-induced neointimal hyperplasia. (A) Quantification of the thickness of neointima 28 days after wire injury. LC: littermate control mice (n=13); KO: myeloid MR knockout mice (n=14). (B) Representative immunofluorescence staining of SMA and Ki67 using cross sections of femoral arteries from sham groups 28 days after operations (n=5). Ki67 was not detectable. (C) Quantification of SMA positive cells. (D) Representative immunofluorescence staining of CD31 using cross sections of femoral arteries 21 days after sham operation or wire injury (injured) (n=5). (E) Immunofluorescence staining of cross sections of femoral arteries 21 days after wire injury using mouse or rat IgG as the primary antibody (negative control). All scale bars: 200 µm.
Supplemental Figure II. Immunohistochemical staining of cross sections of femoral arteries 21 days after wire injury using rabbit IgG as the primary antibody (negative control).
Supplemental Figure III. Flow cytometry analysis of peripheral blood from LC and KO mice 14 days after wire injury. (A) Representative plots of flow cytometry analysis. (B) Quantification of neutrophils, total monocytes, Ly6C<sup>high</sup> and Ly6C<sup>low</sup> monocytes (n=5). ns: not significant.
Supplemental Figure IV. Effects of MR deficiency on expression of genes related to macrophage migration. QRT-PCR analysis of MCP1 and OPN gene expression in peritoneal macrophages (n=6). L32 was used as an endogenous control.
Supplemental Figure V. Immunofluorescence staining of CD68 and BrdU in femoral arteries from LC or KO mice 21 days after wire injury. (A) Representative immunofluorescence staining. Inset images: illustration of boxed areas in high magnification. Scale bar: 200 µm. (B) Quantification of CD68 positive cells and CD68/BrdU double positive cells (n=5).
**Supplemental Figure VI.** Representative immunofluorescence staining of Mac2 and Ki67 using cross sections of femoral arteries from sham groups 28 days after operations (n=5). Neither Mac2 nor Ki67 was detectable.
Supplemental Figure VII. Effects of MR deficiency on expression of genes related to macrophage proliferation. (A) QRT-PCR analysis of IL-4 gene expression in femoral arteries from LC or KO mice 7 days after wire injury or sham operation. GAPDH was used as an endogenous control. ns: not significant. (B) QRT-PCR analysis of M-CSF gene expression in peritoneal macrophages (n=6). L32 was used as an endogenous control.
Supplemental Figure VIII. Effects of conditioned media from MR deficient macrophages on gene expression in VSMCs and MR deficiency on gene expression in macrophages. (A) QRT-PCR analysis of TNFα and MCP1 gene expression in VSMCs treated with macrophage CM or LPS CM for 6 hours. GAPDH was used as an endogenous control. (B) LPS concentration in macrophage CM. Limulus amebocyte lysate (LAL) test was used to measure the concentration. (C) Concentration of IL-6 and IL-1β in CM or LPS CM. (D) QRT-PCR analysis of proinflammatory gene expression in peritoneal macrophages treated with or without LPS (100ng/ml) for 24 hours. L32 was used as an endogenous control.
Supplemental Figure IX. MR deficiency inhibits LPS-induced nuclear translocation of NF-κB p50 in peritoneal macrophages. (A) Representative immunofluorescence staining of nuclear translocation of NF-κB p50 induced by LPS. Scale bar: 200 µm. (B) Quantification of nuclear p50 positive cells.
Supplemental Figure X. SGK1 deficiency decreases proinflammatory gene expression and impairs AP1 and NF-κB signaling pathway in macrophages. (A) QRT-PCR analysis of SGK1 gene expression in peritoneal macrophages from SGK1 knock out (SGK1-KO) mice littermate control (SGK1-LC) mice. (B) QRT-PCR analysis of proinflammatory genes expression in SGK1-KO and SGK1-LC peritoneal macrophages treated with LPS (100ng/ml) or without (-) for 24 hours. (C) Western blotting analysis of phosphorylation and expression of c-Fos and c-Jun in peritoneal macrophages treated with LPS for different time periods. (D) Quantification of (C). (E) Western blotting analysis of phosphorylation and expression of IKKβ and IκBα in peritoneal macrophages treated with LPS. (F) Quantification of (E). (G) Representative immunofluorescence staining of nuclear translocation of NF-κB p65 induced by LPS. The inset images are higher magnification of the boxed areas. Scale bar: 200 μm. (H) Quantification of nuclear p65 positive cells. (I) Representative
immunofluorescence staining of nuclear translocation of NF-κB p50 induced by LPS. Scale bar: 200 µm. (J) Quantification of nuclear p50 positive cells. (K) Western blotting analysis of phosphorylation and expression of p65 in peritoneal macrophages treated with LPS. (L) Quantification of (K).
Supplemental Figure XI. Verification of MR knockdown and SGK1 overexpression in RAW264.7 cells. (A) QRT-PCR analysis of MR gene expression in RAW264.7 cells treated with NC siRNA or MR siRNA. (B) Western blotting analysis of HA tag and phosphorylation of SGK1 in RAW264.7 cells with overexpression of EGFP or constitutively active rat HA-SGK1 (SGK1-OV).