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The role of the mineralocorticoid receptor in the vasculature

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Abstract

Since the mineralocorticoid receptor (MR) was cloned 30 years ago, it has become clear that MR is expressed in extra-renal tissues, including the cardiovascular system, where it is expressed on all cells of the vasculature. Understanding the role of MR in the vasculature has been of particular interest as clinical trials show that MR antagonism improves cardiovascular outcomes out of proportion to changes in blood pressure. The last 30 years of research have demonstrated that MR is a functional hormone-activated transcription factor in vascular smooth muscle cells and endothelial cells. This review summarizes advances in our understanding of the role of vascular MR in regulating blood pressure and vascular function and contributing to vascular disease. Specifically, vascular MR contributes directly to blood pressure control and to vascular dysfunction and remodeling in response to hypertension, obesity and vascular injury. The literature is summarized with respect to the role of vascular MR in conditions including: pulmonary hypertension; cerebral vascular remodeling and stroke; vascular inflammation, atherosclerosis and myocardial infarction; acute kidney injury; and vascular pathology in the eye. Considerations regarding the impact of age and sex on the function of vascular MR are also described. Further investigation of the precise molecular mechanisms by which MR contributes to these processes will aid in the identification of novel therapeutic targets to reduce CVD-related morbidity and mortality.

Introduction

The mineralocorticoid receptor (MR) was first cloned in 1987 (Arriza *et al.* 1987) as a critical regulator of blood pressure through modulation of renal sodium handling in response to its ligand, the steroid hormone aldosterone (Rogerson & Fuller 2000, Rossier *et al.* 2002, Rossier *et al.* 2013). Since that time, substantial investigation has uncovered a myriad of additional roles for MR in health and disease states through its role in the kidney as well as its characterization in extra-renal tissues. Over the past several decades, it has become evident that MR is also expressed in the vasculature, with roles in regulating vascular function and contributing to cardiovascular physiology and pathophysiology. Investigation into the role of MR in the vasculature intensified in the early part of the 21st century after multiple large heart failure trials provided evidence that there may be extra-renal mechanisms of cardiovascular benefits of MR antagonism (Pitt B *et al.* 1999, Pitt B *et al.*

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Conflicts of interest

None.

2001). Additionally, patients with hypertension treated with an MR antagonist have greater protection from end-organ damage than those treated to the same target blood pressure with other classes of anti-hypertensive agents (White *et al.* 2003).

This review summarizes the scientific advances over the past thirty years in our understanding of the role of vascular MR in controlling vascular function and promoting vascular disease. The text specifically highlights the expression and function of MR in the vasculature as well as the *in vivo* role for MR in vascular pathology. We also discuss the creation of transgenic mice in which MR expression is genetically modulated in a vascular cell type-specific manner, resulting in many new and exciting findings that have substantially advanced our understanding of the role of vascular MR in physiology and disease.

MR Expression and Function in the Vasculature

MR is Expressed in Vascular Cells

The blood vessel is composed of 3 layers; the inner intima, consisting of a monolayer of endothelial cells (EC) in contact with circulating blood; the medial layer, composed of vascular smooth muscle cells (SMC) that regulate vessel diameter; and the adventitial layer, composed of fibroblasts and extracellular matrix. Some of the first evidence to suggest the existence of vascular MR was published in the 1980s, even before the cloning of the receptor, in studies demonstrating the presence of specific, high affinity binders (receptors) of mineralocorticoids in femoral and carotid arteries (Kornel, 1981) and in the cytosol of rabbit aorta (Kornel *et al.* 1982a). These early studies also provided evidence that the receptors formed complexes with the mineralocorticoid hormones, which then translocate to the cell nucleus and bind to specific sites on nuclear chromatin (Kornel *et al.* 1982b, Kornel *et al.* 1984). In the 1990s, after the receptor was identified, Lombes *et al.* used anti-MR antibodies to demonstrate that the receptor is present in EC and SMC of the aorta and pulmonary arteries in rabbits (Lombes *et al.* 1992). Several other studies followed, confirming expression of MR also in human pulmonary artery EC and SMC (Hatakeyama *et al.* 1994) as well as in the intact human aorta (Kaye-Wandover *et al.* 2000). This was followed by studies showing that MR functions as a transcriptional regulator in human vascular SMC (Jaffe & Mendelsohn 2005), where it regulates genes involved in vascular fibrosis, inflammation, and calcification (Jaffe & Mendelsohn 2005, Jaffe *et al.* 2007) and in human coronary endothelial cells, where it regulates genes that contribute to inflammation and oxidative stress (Caprio *et al.* 2008).

MR Activation in the Vasculature

There are two endogenous ligands that bind to MR in humans, the mineralocorticoid, aldosterone and the glucocorticoid, cortisol (Arriza *et al.* 1987). Extensive investigation addressing the fact that glucocorticoids circulate at much higher levels than mineralocorticoids ultimately revealed that tissues responsive to aldosterone express the cortisol-inactivating enzyme 11-beta-hydroxysteroid dehydrogenase type 2 (11 β HSD2) (Funder *et al.* 1988). Several recent studies have demonstrated the presence of functional 11 β HSD2 in vascular SMC, further supporting that the vasculature is an aldosterone-

responsive tissue (Kornel 1994, Brem *et al.* 1998, Alzamora *et al.* 2000, Christy *et al.* 2003, Jaffe & Mendelsohn 2005, Caprio *et al.* 2008).

In addition to the classical ligands known to activate MR, several signaling pathways have been implicated in mediating ligand-independent activation of MR. Jaffe and Mendelsohn showed that SMC-MR could be activated not only by aldosterone, but also by Angiotensin II (AngII) via the AngII type 1 receptor (AT1R) (Jaffe & Mendelsohn 2005). This study demonstrated that AngII causes nuclear localization of MR and activation of MR driven gene transcription, and that MR blockade inhibits AngII-induction of MR reporter gene expression in vascular SMC. Further, Lemariè *et al.* demonstrated that aldosterone-induced activation of SMC signaling pathways such as ERK1/2, JNK, and NF- κ B require a functional AT1R (Lemariè *et al.* 2009). The precise mechanisms of the crosstalk between AT1R and MR signaling in response to AngII and aldosterone are still being explored. As the AT1R mediates AngII activation of aldosterone synthase in the adrenal gland, the possibility of local production of aldosterone by SMCs has been considered. While originally controversial, Jaffe and Mendelsohn found no evidence to support local aldosterone production as the mediator of AngII-induced MR activation in human SMC using multiple approaches (Jaffe & Mendelsohn 2005). Thus, alternative mechanisms of MR transactivation by AngII may include ligand-independent activation of MR by post-translational modifications such as phosphorylation, which has been demonstrated for other steroid hormone receptors (Kato *et al.* 1995, Galigniana 1998). Thus, although the mechanisms are not totally elucidated, there is clearly substantial crosstalk between MR and AT1R signaling in the vasculature with important clinical implications, as both drive cardiovascular disease (Rautureau *et al.* 2011).

In addition to AngII, recent studies have also identified a role for the Rho family small GTPase, Rac1, in ligand-independent MR activation in the kidney (Shah *et al.* 2014) and the heart (Nagase *et al.* 2013, Ayuzawa *et al.* 2016). Specifically, *in vitro* studies have shown that overexpression of Rac1 in rat cardiomyocytes promotes nuclear accumulation of MR and increases the transcriptional activity of MR (Nagase *et al.* 2012). In a chronic pressure overload mouse model of heart failure, Rac1 activation increased the expression of MR protein and MR target genes in the heart, which were both reversed by systemic MR blockade, pharmacological Rac1 inhibition and genetic deletion of Rac1 in cardiomyocytes (Ayuzawa *et al.* 2016). More recently, Rac1-mediated MR signaling was implicated in ischemia reperfusion-induced acute kidney injury (Barrera-Chimal *et al.* 2017). There is also limited clinical evidence to suggest the regulation of MR by Rac1 may be important in human disease as Rac1 expression was positively associated with MR expression in humans with high sodium intake, suggesting that Rac1-MR signaling may be involved in high-sodium induced cardiovascular damage (Tapia-Castillo *et al.* 2015). Nonetheless, a precise role for Rac1 in activating vascular MR has yet to be elucidated and further studies are warranted to explore this potential regulation.

MR Regulates Genes in the Vasculature

The use of gene expression profiling confirmed that MR transcriptionally regulates genes in the vasculature and has begun to elucidate the specific genes and pathways involved. The

earliest study in 2005 used tiled glass arrays to determine that MR activation in human coronary artery SMC regulates genes involved in vascular fibrosis, calcification, and inflammation, including: collagen types I and III, the parathyroid hormone receptor, bone morphogenic protein 2, and interleukin-16 (Jaffe & Mendelsohn 2005). The next study used affymetrix gene array technology to profile aldosterone-regulated genes in whole mouse aortas treated ex vivo with hormone (Newfell *et al.* 2011). The microarray results identified 72 aldosterone-regulated genes in the aorta, with overrepresentation of genes involved in oxidative stress, vascular cell proliferation, and extracellular matrix dynamics. These results demonstrated that not only does aldosterone directly regulate vascular genes involved in disease, but that the identity of the MR target genes and the degree of regulation is modified by the conditions to which the vessel is exposed, including the presence of laminar versus turbulent blood flow and the degree of oxidative stress. This concept of context-dependent regulation of vascular function by MR has become a common theme as our understanding of vascular MR function has evolved.

Recently, a role for microRNAs (miRs) in vascular function and disease development has also been recognized (Bonauer *et al.* 2010). miRs are small, non-coding RNAs that regulate expression of groups of proteins through a complementary mRNA seed sequence (a complementary sequence that is essential for miRNA-mRNA binding), resulting in mRNA degradation or inhibition of translation. The role of vascular MR in the regulation of miR expression is just beginning to be explored. Bretschneider *et al.* treated human aortic SMC with physiological levels of aldosterone and performed a microRNA microarray (Bretschneider *et al.* 2016). This study identified miR-29b as an aldosterone-regulated miR in SMCs. Expression of miR29b was reduced by aldosterone treatment in mouse aorta and vascular SMC, but interestingly, not in endothelial cells. In vitro studies further showed that MR blockade with eplerenone prevented the down-regulation of miR-29b in SMC. Thus, it appears that MR regulation of miR expression may be dependent on the specific vascular cell type and that down-regulation of miRs may be an important MR function in the vessel. Although the physiological effects of the down-regulation of miR-29b by SMC-MR are not yet clear, miR-29b has been shown to play a role in vascular aging and in the development of aortic aneurysms (Boon *et al.* 2011) and MR inhibition has been shown to be protective for aortic aneurysm progression by mechanisms that are still unclear (Kurobe *et al.* 2013).

To investigate the direct role of SMC-MR in miR regulation in the context of the aging vasculature, miRNA expression profiling was recently performed in whole mouse aortas from mice that specifically lacked MR from the SMC (DuPont *et al.* 2016). The role of SMC-MR in aging was considered by comparing aortic miRNA expression from young (3 month old) and aged (12 month old) MR-intact and SMC-MR knockout (SMC-MR-KO) mice. This analysis identified miR-155 as the most down-regulated miRNA in the aging vasculature and as a target of SMC-MR (DuPont *et al.* 2016). In vitro reporter assays showed that the miR-155 host gene promoter is transcriptionally inhibited by MR in a ligand-independent manner. The precise mechanism of MR-mediated transcriptional suppression of the miR-155 transcription is unclear; however, the promoter has several binding sites for transcription factors, including Nf-KB and AP-1 (Onyeagucha *et al.* 2013), which are known to interact with the MR to regulate gene transcription (Fiebeler *et al.* 2001).

Thus, over the past three decades it has become clear that MR is expressed in all cells of the vasculature, can be activated by aldosterone and by ligand-independent mechanisms, and contributes significantly to regulation of mRNA and miRNA expression in the vasculature. Further studies are needed to understand the mechanisms by which vascular MR regulates gene expression. Some mechanisms that have been identified for specific genes include direct genomic regulation via MR-responsive elements in gene promoters (Marzolla *et al.* 2017), interaction between MR and other transcription factors such as NFkB and AP-1 (Fiebeler *et al.* 2001), non-genomic activation of signaling pathways that impinge on gene regulation, and suppression of miR expression by MR resulting in up-regulation of vascular miR target genes (DuPont *et al.* 2016). The involvement of vascular MR in other epigenetic mechanisms of gene regulation, including DNA-methylation and histone post-translational modification, has not yet been explored. Importantly, the role of MR in regulating vascular gene expression appears to depend on the vascular cell type and the disease state or age of the organism or the vessel. Thus, although advances in gene expression profiling have yielded substantial novel insights about the role of MR in vascular physiology, there are still many more exciting questions to be answered utilizing rapidly advancing genomic technologies.

Vascular MR in Cardiovascular Pathologies

Cell Type-Specific Modulation of MR In vivo Using Cre-Lox Technology

Before discussing the evolving roles for vascular MR in cardiovascular pathologies, we will briefly review the creation of novel mouse models that have unveiled new and exciting roles for MR in the vasculature. These mouse models were created by engineering mice with loxP sites flanking the MR gene via homologous recombination in ES cells (Metzger *et al.* 1995). Specifically, Cre-Lox technology has significantly advanced our understanding of the specific contribution of MR in SMC versus EC to vascular function and disease. Two promoters have been used to generate mice with MR specifically deleted from SMCs (SMC-MR-KO); the smooth muscle actin promoter driving expression of Cre recombinase fused to a mutant estrogen receptor (ERT2) that is activated by tamoxifen (Wendling *et al.* 2009, McCurley *et al.* 2012a) (inducible SMC-MR-KO mouse), and the SM22alpha promoter, in which active Cre recombinase is constitutively expressed in SMC (constitutive SMC-MR-KO mouse) (Galmiche *et al.* 2014, Tarjus *et al.* 2015). Two different promoters have also been used to create mice with MR deleted from ECs (EC-MR-KO); the Tie2 promoter, which drives expression of Cre recombinase in myeloid cells in addition to ECs (Schäfer *et al.* 2013, Rickard *et al.* 2014), and the vascular endothelial (VE)-cadherin Cre promoter, which is specifically expressed in ECs (Barret-Mueller *et al.* 2015, Jia *et al.* 2015, 2016). The development of these novel mouse models along with mice in which human MR is specifically overexpressed in ECs has shed light on the direct and specific role of vascular SMC- or EC-MR in blood pressure control, aging, vascular remodeling, inflammation, cardiac dysfunction, renal disease, and ocular diseases (summarized in Table 1). We will discuss the role of vascular MR in each of these pathologies and the novel insights the vascular cell-specific MR knockout and transgenic mouse models have contributed to our understanding of MR in the vasculature.

Direct Contribution of Vascular MR to Blood Pressure Regulation

The use of MR antagonists in humans and animal models provided initial support for the concept that vascular MR directly contributes to blood pressure regulation. MR antagonism with eplerenone improved brachial artery flow-mediated dilation (FMD), a clinical marker of endothelial function, in hypertensive humans (Fujimura *et al.* 2012). Further, eplerenone treatment reduced BP and pulse pressure as well as vascular stiffness in rats that were treated with aldosterone (Lacolley *et al.* 2002). A meta-analysis of BP trials with eplerenone in humans suggested that the decline in BP in response to MR inhibition did not correlate with the degree of potassium elevation, suggesting a non-renal mechanism was also contributing to the BP benefits of MR antagonism (Levy *et al.* 2004). The first line of evidence demonstrating a direct role for vascular MR in blood pressure control was in 2010; Nguyen Dinh Cat *et al.* generated a mouse model that could be induced to overexpress human MR in ECs (Nguyen Dinh Cat *et al.* 2010). This mouse model exhibited increased blood pressure upon induction of EC-MR overexpression with impaired vascular reactivity, specifically, increased contractile function. In contrast, EC-specific MR deletion does not appear to alter blood pressure in male mice (Schäfer *et al.* 2013, Rickard *et al.* 2014, Barret-Mueller *et al.* 2015), suggesting that while EC-MR may not contribute to BP regulation under basal conditions, a role in BP regulation may be manifest under conditions that induce EC-MR upregulation. Following this study, McCurley *et al.* showed that SMC-MR directly contributes to blood pressure control using the inducible SMC-MR knockout mouse (McCurley *et al.* 2012a). Arterial telemetry studies revealed that SMC-MR-KO mice are protected from the aging-associated increases in blood pressure seen in MR-intact littermates and hence aged mice lacking SMC MR have lower blood pressure (McCurley *et al.* 2012a). Importantly, renal MR function was confirmed to be intact in these mice and the alterations in blood pressure in SMC-MR-KO mice were independent of sodium loading conditions. Further studies showed that SMC-MR-KO mice generated less spontaneous vascular myogenic tone, suggesting that SMC-MR contributes to blood pressure control via alterations in vasoconstriction by changes in vascular L-type calcium channel expression and function (McCurley *et al.* 2012a, DuPont *et al.* 2016). Further mechanistic studies revealed that SMC-MR-KO mice were less responsive to AngII. Specifically, the lack of SMC-MR attenuated AngII-induced vascular oxidative stress, vasoconstriction and hypertension. In this way, this study provided the first in vivo evidence for crosstalk between MR and AngII signaling in SMC, which had been demonstrated in multiple previous studies in cultured SMC as described above (Hatakeyama *et al.* 1994, Jaffe & Mendelsohn 2005, Mazak *et al.* 2004, Rautureau *et al.* 2011, Xiao *et al.* 2004). Furthermore, these data support that this crosstalk has substantial physiologic significance by contributing directly to blood pressure regulation independent of kidney function.

Several additional studies have confirmed a role for SMC-MR in blood pressure regulation and vasoconstriction in mice with constitutive deletion of SMC-MR (Galmiche *et al.* 2014, Tarjus *et al.* 2015). It is important to note that the decrease in blood pressure in this model is evident even in younger mice (4 months old). As MR is constitutively deleted from the time of fetal development in this model, it is possible that the duration of SMC-MR deletion contributes to the timing of the blood pressure phenotype. The constitutive SMC-MR-KO mouse also exhibited blunted contractile responses of conduit vessels (aorta) to potassium

chloride and calcium (Tarjus *et al.* 2015). SMC-MR-KO mice were also found to have decreased phosphorylation of the contractile regulatory proteins myosin light chain kinase (MLCK) and myosin phosphatase-targeting protein subunit 1 (MYPT1) in the aorta. Thus, it is clear that SMC-MR directly contributes to blood pressure regulation, at least in part, by modulating vasoconstriction.

A Role for Vascular MR in Cardiovascular Dysfunction Induced by Risk Factors

Vascular MR in the Response to Hypertension—In a rat model of AngII-induced hypertension, spironolactone treatment reduced vascular complications in part by reductions in oxidative stress (Virdis *et al.* 2002). As mentioned above, EC-specific MR deletion did not affect blood pressure or vascular function in healthy mice, but male EC-MR-KO mice were protected from endothelial dysfunction caused by AngII-induced hypertension (Barrett-Mueller *et al.* 2015). Additionally, Rickard *et al.* reported that deletion of EC-MR prevented DOCA/salt hypertension-mediated vascular inflammation and fibrosis (Rickard *et al.* 2014). Both of these studies support the emerging idea that EC-MR contributes to vascular disease induced by cardiovascular risk factors. One potential explanation for these results could be that EC-MR is neutral or perhaps even protective in healthy states, but that this protection is lost when cardiovascular risk factors are present, as described in an accompanying editorial to those studies (Jaffe & Jaisser, 2014) and reviewed recently elsewhere (Davel *et al.* 2017). In addition, due to the expression of the Tie2 promoter in myeloid cells, it is plausible that leukocyte MR may also influence endothelial function in the presence of risk factors independent of EC-MR. Thus, these studies support the idea that EC-MR does not play a significant role in the control of blood pressure and vascular function in non-disease states; however, in the setting of cardiovascular risk factors, including hypertension, EC-MR contributes to the development of endothelial dysfunction and the ensuing vascular damage.

Similarly, constitutive SMC-MR-KO mice were challenged with hypertension induced by unilateral nephrectomy combined with either vehicle or aldosterone infusion with high salt (1% NaCl) intake (NAS model of hypertension) (Galmiche *et al.* 2014). Although NAS treatment increased blood pressure to a similar level in MR-intact and SMC-MR-KO mice, SMC-MR-KO mice exhibited less vascular stiffness, a measure of vessel remodeling and dysfunction in response to hypertension that correlates with adverse vascular outcomes in humans (Palombo *et al.* 2016).

Vascular MR in the Response to Obesity and Diabetes—Although the recent Treatment of Preserved Cardiac Function Heart Failure with an Aldosterone Antagonist (TOPCAT) trial, a randomized trial of MR inhibition in heart failure with preserved ejection fraction (HFpEF), was negative, there was evidence to suggest that specific patient subgroups with HFpEF may benefit from MR antagonism (Pitt *et al.* 2014, Shah *et al.* 2014). Indeed, several smaller clinical studies have observed improved diastolic function in response to MR antagonism in subpopulations with diabetes, obesity, and metabolic syndrome (Kosmala *et al.* 2011, 2013). Hwang *et al.* provided further clinical evidence to suggest a link between vascular dysfunction in people with obesity and vascular MR, as the

degree of improvement in FMD in response to eplerenone was associated with higher total body fat and abdominal fat in healthy older adults (Hwang *et al.* 2013).

At the preclinical level, the use of MR antagonists has further expanded upon these findings. Bender *et al.* treated obese zucker rats with low dose spironolactone and observed an improvement in coronary endothelial function in response to insulin and acetylcholine (ACh) (Bender *et al.*, 2015). Although aortic endothelium-dependent relaxation was normal in healthy EC-MR-KO mice (Schäfer *et al.* 2013), female EC-MR-KO mice were protected from aortic endothelial dysfunction, vascular stiffness, and cardiac diastolic dysfunction in the setting of Western diet-induced obesity (Jia *et al.* 2015, 2016). There is evidence to support that EC-MR may also contribute to vascular stiffening by a distinct mechanism. The epithelial sodium channel (ENaC) is a classical target of MR in the kidney where it contributes to sodium transport. ENaC was recently shown to be also regulated by MR in ECs. Aldosterone treatment increased expression of ENaC in human ECs *in vitro* in association with increased EC stiffness as measured by atomic force microscopy (Kusche-Vihrog *et al.* 2008, Drüppel *et al.* 2013). This mechanism may also contribute to the role of EC-MR in endothelial dysfunction; ENaC-induced EC stiffening is associated with decreased eNOS activity, resulting in less NO production (Jia *et al.* 2016), a marker of endothelial dysfunction. These studies provide sound evidence that EC-MR contributes to vascular stiffness, especially in the setting of metabolic cardiovascular risk factors, and that this may lead to endothelial dysfunction, an early step in the development of vascular disease. Further, it is likely that there are synergistic effects of vascular MR in CVD-associated pathologies as obesity is a major cause of diabetes and hypertension (Jia *et al.* 2014), such that, in patients with more than one risk factor, the contribution of MR to vascular damage may become heightened.

Vascular MR in the Response to Vessel Injury—In response to injurious stimuli including direct mechanical injury, hypertension, and aging, the vasculature remodels by activation of SMC proliferation, migration, and extracellular matrix deposition. This remodeling results in vascular fibrosis and stiffening that contributes to impaired vessel function and predicts adverse outcomes in humans (Lee *et al.* 2016). Ample studies have demonstrated that aldosterone enhances this pathologic vascular remodeling process and that MR antagonists are protective (reviewed in McCurley & Jaffe 2012). Several studies have recently demonstrated a direct role for SMC-MR in vascular remodeling after injury from mechanical damage. Pruthi *et al.* exposed the inducible SMC-MR-KO mouse model to wire-induced carotid injury and showed that SMC-MR is necessary for aldosterone-induced vascular remodeling *in vivo* (Pruthi *et al.* 2014). Treatment with a dose of aldosterone, which did not increase blood pressure, significantly enhanced the vascular response to injury in MR-intact mice in this model (Jaffe *et al.* 2010). However, SMC-MR-KO mice were protected from aldosterone-induced vascular fibrosis and SMC proliferation (Pruthi *et al.* 2014). Importantly, even in the absence of exogenous aldosterone, vehicle-treated SMC-MR-KO mice had less vascular fibrosis in response to injury compared to vehicle-treated MR-intact littermates. Interestingly, in the uninjured vessel, the presence of SMC-MR or treatment with aldosterone did not affect the degree of vessel thickness or fibrosis (Jaffe *et al.* 2010, Pruthi *et al.* 2014). These results demonstrate that SMC-MR may directly

contribute to vascular remodeling specifically in areas of vascular injury in the presence of physiological or pathological levels of aldosterone. Studies have elucidated several molecular mechanisms by which SMC-MR directly contributes to vascular remodeling, including Rho-kinase signaling, placental growth factor signaling through vascular endothelial growth factor type 1 receptor, integrin upregulation and galectin expression. Each of these specific mechanisms has been recently reviewed in detail elsewhere (Koenig & Jaffe 2014). Importantly, in both of the SMC-MR-KO mouse models, the role for SMC-MR in adverse vascular remodeling was independent of blood pressure (McCurley *et al.* 2012a, Tarjus *et al.* 2015). Overall, these data reveal that neither MR activation with aldosterone nor SMC-MR deletion affect vascular remodeling in the absence of a vascular injury stimulus, further supporting the evolving model in which SMC-MR acts synergistically with vascular injury to contribute to vascular remodeling and stiffness.

Additionally, a role for EC-MR in oxidative stress-induced vascular damage has been suggested by several studies. Diseased vessels produce reactive oxygen species that contribute to enhanced vascular oxidative stress, a well characterized mechanism of endothelial dysfunction and decreased NO bioavailability. Global MR blockade has been shown to increase NO bioavailability via reductions in vascular oxidative stress by reducing eNOS uncoupling and increasing vascular superoxide dismutase and catalase expression (Chen *et al.* 2016). More recently, a specific role for EC-MR in this process was identified. EC-MR deletion in mice prevented the aldosterone-induced increase in superoxide production in cerebral arteries (Dinh *et al.* 2016) and also improved aortic endothelium-dependent vasorelaxation in mice fed a Western diet (Jia *et al.* 2016).

A Role for MR in Pulmonary Hypertension

There is clinical evidence to support a role for MR in pulmonary arterial hypertension (PAH). Spironolactone improved exercise tolerance and decreased plasma brain natriuretic peptide levels (a marker of cardiac dysfunction) in PAH patients (Maron *et al.* 2013). MR antagonism has also been shown to be beneficial in several preclinical animal models of pulmonary hypertension. Preston *et al.* showed that spironolactone attenuated adverse pulmonary arterial remodeling and prevented the rise in right ventricular systolic pressure in a mouse model of hypoxia-induced PAH (Preston *et al.* 2013). In a rat model of monocrotaline-induced PAH, MR inhibition prevented the progression of PAH, even when the drug was initiated after pulmonary pressure was elevated. In this study, it was also shown that MR activation induced SMC proliferation in pulmonary artery SMC, suggesting that MR in the SMC may contribute to PAH. Further in vitro studies have provided evidence that endothelial MR may also be important in PAH. In pulmonary artery endothelial cells, aldosterone induced a sulfenic posttranslational modification of the endothelin receptor type B, leading to reduced nitric oxide bioavailability in the pulmonary vasculature (Maron *et al.* 2012). Additionally, a study reported that hypoxia induced secretion of aldosterone from pulmonary artery endothelial cells and that this might contribute to pulmonary vascular fibrosis (Maron *et al.* 2014). Thus, MR appears to contribute to the development and progression of pulmonary hypertension and vascular MR likely contributes, at least in part, to the mechanism. An ongoing clinical trial is underway to determine whether MR inhibition

could be a novel therapy to prevent progression of PAH ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01712620) Identifier NCT01712620).

MR in Cerebral Vascular Remodeling and Stroke

Clinically, there is a strong link between aldosterone and stroke (Conn *et al.* 1964); patients with primary hyperaldosteronism (Milliez *et al.* 2005) and elevated plasma aldosterone levels have an increased frequency of stroke (McMahon *et al.* 2004) compared with those with hypertension without elevated aldosterone. Preclinical models have implicated a role for MR in the cerebral vasculature in response to stroke. Specifically, spironolactone treatment has been shown to reduce the damage caused by cerebral ischemia in stroke-prone spontaneously hypertensive rats (Dorrance *et al.* 2001). Further, in a DOCA-induced hypertension model in rats, MR activation caused remodeling of the cerebral vasculature, leading to increased vessel stiffness (Dorrance *et al.* 2006). Additional studies demonstrated that MR antagonism with spironolactone increased the vascular tone of the middle cerebral artery (MCA) and improved vascular structure by increasing lumen and outer diameter of the vessel in male (Rigsby *et al.* 2007a) but not female (Rigsby *et al.* 2007b) spontaneously hypertensive stroke-prone rats. In contrast, a recent study showed that MR antagonism during the development of hypertension has opposing effects on the MCA; it simultaneously caused a reduction in lumen diameter, which may be deleterious to vascular function and stroke outcomes, and simultaneously improved vasodilation (McClain & Dorrance 2014). Together, these studies suggest that vascular MR may contribute to cerebral vascular remodeling in response to hypertension thereby contributing to stroke, although the specific vascular cell type remains to be determined. Additionally, this evidence suggests that vascular MR may exert opposing physiological effects, depending upon the time course of vascular injury and on the sex of the individual.

MR Contributes to Atherosclerosis & Myocardial Infarction

Atherosclerosis is a systemic vascular inflammatory disease that is initiated by cardiovascular risk factors leading to endothelial damage and ultimately progressing to plaque development and rupture to cause myocardial infarction or stroke. Clinical data reveal that circulating aldosterone levels are independent predictors of cardiovascular ischemia (Milliez *et al.* 2005, Barter *et al.* 2007) and that primary hyperaldosteronism patients have a 6-fold increased risk of myocardial infarction (MI) even after controlling for blood pressure (Vergeer *et al.* 2008). Additionally, MR antagonists improve clinical outcomes in patients with heart failure after myocardial infarction (MI) (Pitt *et al.* 2001, 2003). Thus, there is strong a clinical link between MR activation and atherosclerosis complications. Here we describe recent in vitro studies and preclinical studies in animal models that have begun to explore the mechanism for this link.

Early evidence specifically connecting vascular MR to the mechanism of vascular inflammation came from Caprio *et al.* in 2008. This study demonstrated that aldosterone, acting through EC-MR, promotes expression of the cell adhesion molecule, intracellular adhesion molecule-1 (ICAM-1), thereby promoting leukocyte adhesion to human coronary ECs in vitro (Caprio *et al.* 2008). Further in vitro studies revealed that this was mediated by a direct effect of MR on ICAM-1 transcription via an MR responsive element in the ICAM-1

promoter (Marzolla *et al.* 2017). MR regulation of ICAM-1 and the resulting increase in leukocyte adhesion to ECs in vitro was inhibited by the estrogen receptor probably via formation of a complex with the MR in the cell nucleus (Barrett-Mueller *et al.* 2014). These studies provide a potential mechanism that could contribute to sex differences in cardiovascular ischemia.

Leukocyte-EC adhesion is the first step in the process of leukocyte recruitment from the circulation into the vessel wall to produce vascular inflammation, an early step in atherosclerosis, and for tissue inflammation in response to injury or infection. In vivo, in the apolipoprotein E knockout model of atherosclerosis, aldosterone infusion at a dose that did not alter blood pressure accelerated the burden of atherosclerosis and produced plaques with increased inflammation, a phenotype prone to rupture and cause myocardial infarctions in humans (McGraw *et al.* 2013). This was prevented in mice genetically deficient in ICAM-1, demonstrating that ICAM-1 is necessary for aldosterone -induced atherosclerotic plaque formation and inflammation in this model (Marzolla *et al.* 2017).

Several studies have explored the potential role of EC-MR in cardiac inflammation. Using the Tie2-Cre model of EC-MR deletion, one study showed that EC-MR-KO mice had decreased DOCA/Salt-induced cardiac inflammation with decreased cardiac macrophage recruitment and fibrosis (Rickard *et al.* 2014). Whether this is due to a role for MR in the EC or in the macrophage cannot be determined using this model. To further address this question, a recent study utilized the VE-Cad-Cre model. Lothar *et al.* confirmed that DOCA/salt-induced expression of vascular cell adhesion molecular (VCAM-1) is mediated by EC-MR and this leads to cardiac remodeling and inflammation (Lothar *et al.* 2016). These effects were prevented in EC-MR-KO mice. In addition, another recent study examined cardiac inflammation in response to pressure overload induced by trans-aortic constriction. Salvador *et al.* showed that pressure overload causes ICAM-1 upregulation in the heart which is necessary for cardiac inflammation and fibrosis (Salvador *et al.* 2016). However, ICAM-1 upregulation and cardiac inflammation was not affected by specific deletion of MR from ECs using the VE-Cad-Cre model. Overall, studies support that EC-MR contributes to cardiovascular inflammation in response to atherogenic and hypertensive stimuli by promoting expression of leukocyte adhesion molecules on ECs. Further studies are needed to clarify the specific adhesion molecules and the exact role of EC-MR, as these mechanisms may depend on the vascular bed and the vascular injury stimulus.

A role for SMC-MR in myocardial infarction has also recently been explored. Gueret *et al.* demonstrated that SMC-MR contributes to coronary and left ventricular dysfunction in a mouse model of MI (Gueret *et al.* 2016). This study elegantly compared the cardiac and vascular response to left coronary artery ligation in MR-intact compared to constitutive SMC-MR-KO littermates. Specifically, SMC-MR-KO mice exhibited less cardiac interstitial fibrosis and improved diastolic function after MI compared to MR-intact littermates. This was associated with improved coronary vascular relaxation in SMC-MR-KO mice due to decreased coronary oxidative stress in mice lacking SMC-MR. A role for cardiomyocyte-MR in left ventricular dysfunction and remodeling after MI has been previously demonstrated using a similar model (Fraccarollo *et al.* 2011). Taken together, these studies support that both SMC-MR and cardiomyocyte-MR contribute to cardiac dysfunction and

fibrosis after MI, and may thereby contribute to the benefits of MR antagonist therapy in post MI patients (Pitt *et al.* 2001).

Vascular MR in Acute Kidney Injury

Acute kidney injury (AKI) is a risk factor for chronic kidney disease and a common complication in hospitalized patients that currently has no effective prescribed treatment. Several recent studies have indicated a role for MR in AKI by demonstrating that MR antagonism reduces ischemia reperfusion injury in a rat model of AKI (Barrera-Chimal *et al.* 2016, Lattenist *et al.* 2017). A specific role for SMC-MR was recently explored in a model of renal dysfunction in response to Cyclosporine A (CsA). CsA is widely used after renal transplantation as immunosuppressant therapy, with a side effect of acute nephrotoxicity in some patients (Amador *et al.* 2016). Amador *et al.* exposed the constitutive SMC-MR-KO mouse to CSA-induced acute kidney failure and found that SMC-MR-KO mice were protected from CsA-induced kidney dysfunction, tubular vacuolization, and neutrophil gelatinase-associated lipocalin (NGAL) upregulation, a novel biomarker of kidney damage. This protection was attributed to blunted vascular L-type calcium channel activity in SMC-MR-KO mice resulting in decreased renal artery vasoconstriction and overall improvement in renal hemodynamics. Of note, EC-MR deletion did not have any effect on CsA-induced renal damage, suggesting that the deleterious effects of such an insult are mediated via SMC-MR only. Similarly, a very recent study addressed the potential role of SMC-MR and EC-MR in AKI induced by ischemia reperfusion. Barrera-Chimal *et al.* subjected SMC-MR-KO and EC-MR-KO mice to renal ischemia followed by reperfusion and found that deletion of MR from SMC but not EC prevented ischemia/reperfusion-induced renal damage (Barrera-Chimal *et al.* 2017). This study further utilized the Large White pig to translate these findings to a large preclinical animal model with more similar renal structure to humans. Pigs that were treated with MR antagonist canrenoate had improved renal outcomes after being subjected to bilateral renal ischemia. Together, these studies support the notion that MR antagonist therapy may be beneficial in clinical trials of acute kidney injury, a condition in which there are currently no known effective treatments, and this may be mediated by inhibition of SMC-MR in the renal vasculature.

MR in the Vasculature of the Eye

MR has been shown to be expressed in cells of the eye including retinal cells, glia, and ECs. Neovascularization in the eye is an important cause of visual impairment in premature infants and in patients with diabetes. MR has recently been shown to contribute to neovascularization in the eye. Although the mechanisms are not totally clear, EC-MR appears to contribute. In a rat model of retinopathy of prematurity, retinal MR, AT1R, and ENaC expression were decreased in response to a low salt diet (Deliyanti *et al.* 2014). Further, high salt treatment increased MR, AT1R and ENaC expression in cultured retinal cells, which was prevented with an MR antagonist. These results suggest that alterations in expression of RAAS components are an essential aspect of low salt diet-mediated protection from retinopathy. Another study in a similar rat model reported that EC-MR activation in response to local production of aldosterone by glial and ganglion cells promoting expression of inflammatory genes and contributes to neovascularization in neonates (Deliyanti *et al.* 2012).

A role for EC-MR in central serous chorioretinopathy (CSCR), a condition in which there is a focal disruption of the outer retinal barrier and subretinal serous fluid accumulation leading to visual loss (Gemenetzi *et al.* 2010), is an example of how such exploration can lead to breakthroughs in therapy. Zhao *et al.* challenged rats with an intravitreal injection of corticosterone, which induced choroidal enlargement in the eyes, essentially creating a rat model of CSCR (Zhao *et al.* 2012). The authors found that aldosterone injections had a similar effect in mimicking CSCR and that aldosterone-induced upregulation of the ion channel KCa2.3 in ECs was an underlying mechanism for this condition. Further, MR antagonism reversed the aldosterone-induced choroidal thickening and upregulation of KCa2.3, supporting that MR in choroidal vessels contributes to ocular pathologies. These findings were then translated to humans in a small clinical study. Short term MR antagonism successfully resolved CSCR in two patients with persistent CSCR. Since that original study, more patients have been treated with MR antagonists for CSCR with improvement in vision supporting MR antagonism as a novel therapy for this otherwise debilitating condition (Ghadiali *et al.* 2016).

Aging & Sex Differences in Vascular MR Function

MR in Vascular Aging

Aging is universal and a prominent cardiovascular disease risk factor. Vascular aging is a multi-faceted process that involves changes in vessel structure and function, yet the precise molecular mechanisms that contribute to vascular aging are not fully understood. A role for MR in vascular aging was suggested by a study in 2010 in which Krug *et al.* demonstrated that MR expression and signaling increase with age in rat vessels, specifically in the SMC (Krug *et al.* 2010). More recently, DuPont *et al.* confirmed the rise in MR expression with aging in resistance vessels and characterized the specific role of SMC-MR in changes in vascular function with aging in vivo using SMC-MR-KO mice (DuPont *et al.* 2016). As described previously, gene expression profiling in vessels from these mice identified miR-155 as a transcriptionally-repressed target of SMC-MR and the most down-regulated miRNA in the aging vasculature. Furthermore, miR-155 was found to target pro-constrictive genes including Cav1.2, the pore-forming subunit of the L-type calcium channel, and the AT1R (DuPont *et al.* 2016). Thus, it was revealed that as mice age, vascular MR expression rises and miR155 decreases, and as a result, expression of calcium channels and AT1 receptors increase, contributing to an increase in vasoconstriction, vascular tone, oxidative stress and ultimately blood pressure. These effects of aging were all attenuated in mice specifically lacking SMC-MR. Moreover, restoration of miR-155 in SMC of aged MR-intact mice by a SMC-targeted lentivirus, reversed the aging-associated changes in gene expression, vasoconstriction, and oxidative stress. miR155 has previously been found to decrease with aging in human leukocytes (Noren Hooten *et al.* 2010) and a SNP in the miR155 target sequence of the AT1R gene that prevents miR155 binding is associated with hypertension in humans (Ceolotto G *et al.* 2011). Finally, a very small clinical study showed that when aged humans were treated for one month with the MR antagonist eplerenone, a rise in serum miR-155 in response to MR inhibition was associated with significantly greater reduction in both systolic and diastolic blood pressure (DuPont *et al.* 2016). These studies provide a molecular mechanism for the role of SMC-MR in vasoconstriction, blood pressure

regulation, and aging. However, larger studies are warranted in order to confirm this clinical finding, that serum miR-155 may be a useful biomarker to identify elderly people most likely to benefit from MR antagonist therapy. In addition to vascular tone, constriction, and blood pressure, there are several other markers of vascular aging such as DNA damage, apoptosis, and senescence. The specific role of vascular MR in these various aging mechanisms has yet to be elucidated.

Sex Differences in the Role of Vascular MR

Several recent studies support that there are sex differences in the role of EC-MR in vascular dysfunction in response to cardiovascular risk factors. It has been shown that obesity is associated with increases in plasma aldosterone (Bentley-Lewis *et al.* 2007), probably due to adipocyte production of aldosterone-releasing factors, including leptin (Huby *et al.* 2015). Interestingly, obese female mice exhibit higher plasma aldosterone levels than their obese male counterparts (Jia *et al.* 2016) and MR antagonism improves endothelial function to a greater degree in hyperleptinemic female mice compared to males (Huby *et al.* 2016). In addition, female EC-MR-KO mice are protected from Western-diet induced vascular stiffness and endothelial dysfunction (Jia *et al.* 2016), although males have not been tested. These studies support that the role of EC-MR in promoting vascular dysfunction in response to risk factors or vascular damage may depend on sex, although, the detailed molecular mechanisms remain to be determined. One potential mechanism that merits exploration in vivo involves estrogen receptor inhibition of MR transcriptional activity which has been demonstrated in vitro in human coronary endothelial cells (Barrett-Mueller *et al.* 2014). However, additional studies are warranted before a clear role of sex differences in EC-MR mediated vascular dysfunction can be identified. Further, the majority of the preclinical studies described in this review were performed in males only, with almost no studies directly comparing the role of MR in both sexes side by side, and this remains an important limitation to the generalizability of the findings.

Summary

Since the cloning of MR 30 years ago, substantial progress has been made in our understanding of the role of MR in the vasculature and its contribution to vascular disease. The initial identification of vascular MR expression led to further exploration using gene expression profiling and revealed that MR is a functional transcription factor in vascular SMC and EC. Further studies utilizing MR antagonists provided evidence of an in vivo role for MR in vascular function in a variety of CVD-associated pathologies at both the preclinical and clinical level. Over the past decade, Cre-Lox and transgenic mouse technology has enabled the characterization of cell-type specific roles for SMC- and EC-MR, independent of renal MR activity and function. It is clear there are distinct roles for MR in SMC versus EC. SMC-MR directly contributes to blood pressure control and vascular tone by regulating calcium channel expression and mediating AngII signaling which is further enhanced with aging. SMC-MR also contributes to vascular remodeling specifically in response to hypertension or mechanical injury (Figure 1). EC-MR does not appear to play a major role in baseline blood pressure control or basal vasomotor function. However, in the setting of cardiovascular risk factors including hypertension and obesity, EC-MR contributes

significantly to endothelial dysfunction and vascular damage, probably through mechanisms involving oxidative stress, inflammation, and vessel stiffening (Figure 2). Taken together, the studies demonstrate that vascular MR contributes to systemic and pulmonary hypertension, cardiac and kidney damage, cerebrovascular remodeling and stroke, common disorders of the eye, and atherosclerosis and vascular inflammation, providing many new opportunities that may benefit from MR antagonists. There are also studies which suggest that there may be sex differences in the role of vascular MR in the setting of cardiovascular risk factors, but further studies directly comparing males and females are needed to fully elucidate the mechanisms involved. The past several decades of research have truly expanded our knowledge and understanding of MR in the vasculature. However, further exploration of the precise mechanisms by which vascular MR contributes to vascular disease is essential to determining novel therapeutic interventions to reduce the cardiovascular disease burden in at-risk populations.

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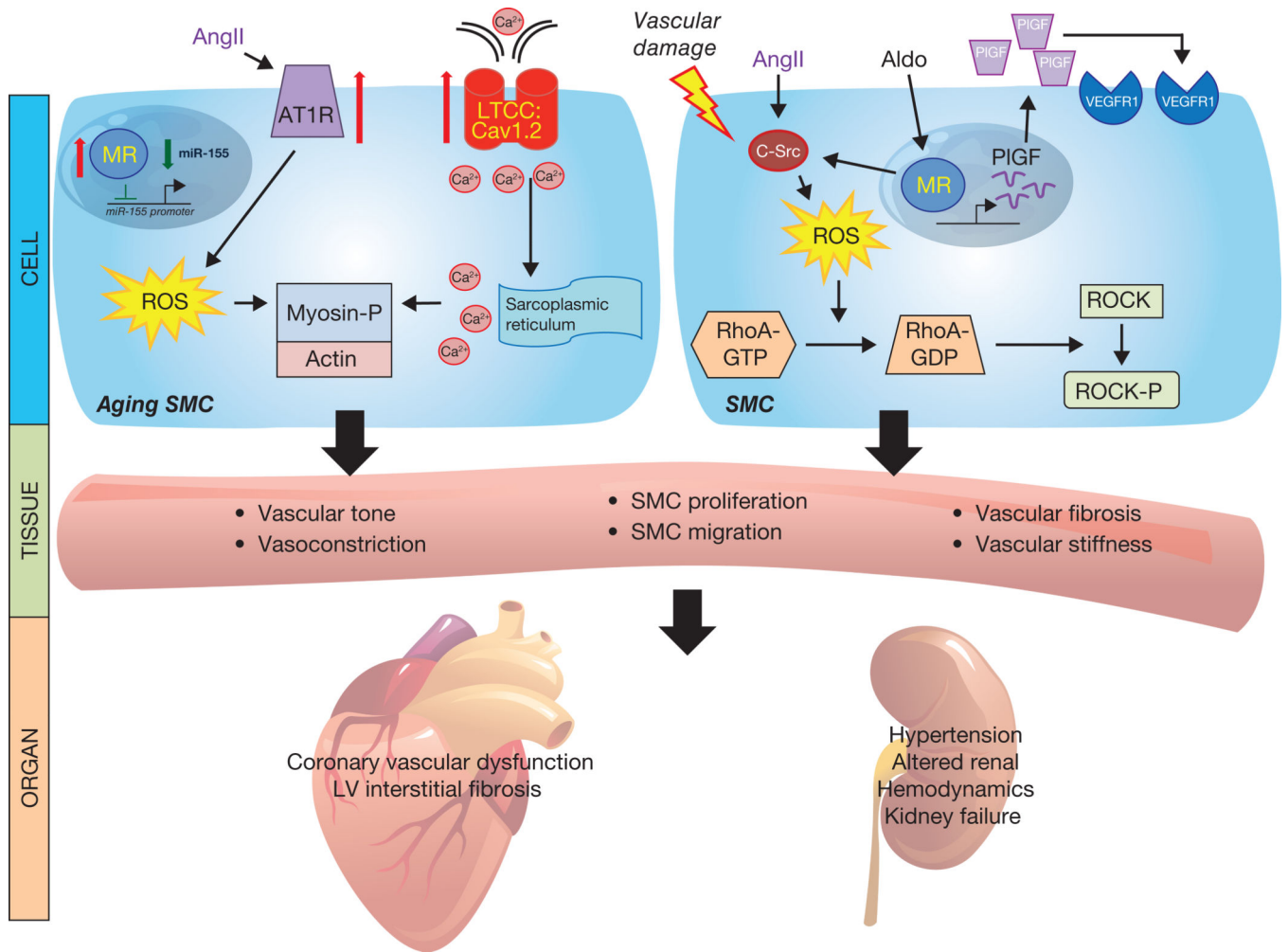


Figure 1. Model for the role of smooth muscle cell-mineralocorticoid receptor (SMC-MR) in vascular function

At the cellular level, SMC-MR contributes to vascular tone and vasoconstriction, specifically in aging SMC. MR expression rises with age, resulting in suppressed transcription of miR-155, leading to the up-regulation of the Ang II type 1 receptor (AT1R) and L-type calcium channel (LTCC) subunit Cav1.2. SMC-MR contributes to vascular remodeling via several mechanisms, including: (1) Rho-kinase signaling, which is activated by aldosterone (Aldo) via SMC-MR or Ang-II, leading to c-src and Rho-associated kinase activation and SMC migration; (2) Placental growth factor (PIGF) signaling leading to vascular remodeling specifically in areas on vascular damage/injury, where the vascular endothelial growth factor type 1 receptor (VEGFR1) is up-regulated by SMC-MR. Together, these effects of SMC-MR activation lead to significant effects on vessel function, including: increases in vascular tone, vasoconstriction, SMC proliferation, SMC migration, vascular fibrosis, and vascular stiffness. Ultimately, SMC-MR activation results in end-organ damage such as high blood pressure, coronary vascular dysfunction, cardiac interstitial fibrosis, alterations in renal hemodynamics, and kidney failure. Ang II; Angiotensin II. ROS; reactive oxygen species. ROCK; Rho-associated kinase. Ca; calcium.

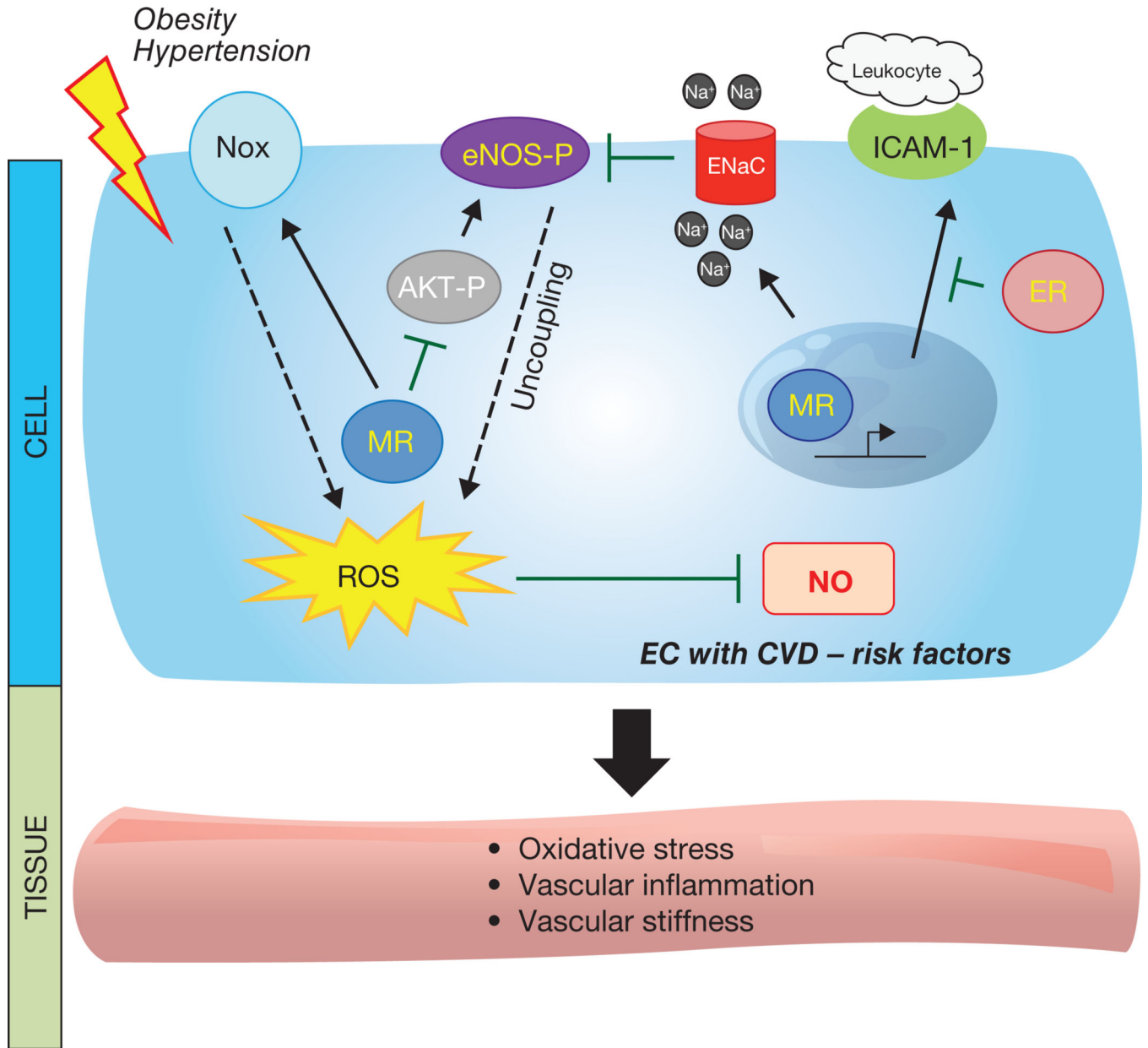


Figure 2. Model for the role of endothelial cell-mineralocorticoid receptor (EC-MR) in vascular function

In the presence of cardiovascular disease risk factors, such as diabetes, obesity, and hypertension, EC-MR contributes to endothelial dysfunction via endothelial nitric oxide synthase uncoupling, NADPH oxidase activation, increases in epithelial sodium channel expression, and inflammation via ICAM-1-mediated leukocyte adhesion. ICAM-1; intracellular adhesion molecule-1. ROS; reactive oxygen species. NO; nitric oxide. eNOS; endothelial nitric oxide synthase. ENaC; epithelial sodium channel.

Table 1

Pathology	Cell Type	Role of MR Activation	References
<i><u>Blood Pressure control/HTN</u></i>	EC	Overexpression increases blood pressure; knockout has no effect	8, 86, 97, 104
	SMC	Increases blood pressure	38, 77, 108
<i><u>Response to HTN</u></i>	EC	Endothelial dysfunction Vascular inflammation & fibrosis	8, 97
<i><u>Response to Obesity</u></i>	EC	Endothelial dysfunction & vascular stiffness Cardiac diastolic dysfunction	47, 48, 97
<i><u>Pulmonary HTN</u></i>	Unclear	Pulmonary vascular remodeling & increase right ventricular systolic pressure	72, 94
<i><u>Cerebral vascular remodeling & Stroke</u></i>	Unclear	Vascular remodeling & vascular stiffness Lumen diameter reduction	25, 76, 98
<i><u>Atherosclerosis & Myocardial Infarction</u></i>	EC	Upregulation of ICAM-1 & VCAM-1; Leukocyte-EC adhesion	16, 69
	SMC	Endothelial dysfunction & oxidative stress, Cardiac interstitial fibrosis & Left ventricular dysfunction	30, 37
<i><u>Acute Kidney Injury</u></i>	SMC	Increases LTCC activity Increases vasoconstriction	2, 5
<i><u>Retinal Vasculature</u></i>	SMC	Choroidal vessel thickening & increased expression of KCa2.3	114
<i><u>Aging</u></i>	SMC	Increases BP through increases in LTCC activity, vasoconstriction, myogenic tone & oxidative stress	28, 59, 77

MR; mineralocorticoid receptor. HTN; hypertension. EC, endothelial cell. SMC; smooth muscle cell. ICAM-1; intracellular adhesion molecule. VCAM-1; vascular cell adhesion molecule. LTCC; L-type calcium channel. CSCR; central serous chorioretinopathy.