Chapter 4

Mineralocorticoid Receptor in Calcium Handling of Vascular Smooth Muscle Cells

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Abstract

For decades, the mineralocorticoid receptor (MR) antagonists have been used for the management of cardiovascular diseases; however, the molecular mechanisms involved in their beneficial effects are not fully understood. Recent publications point to the fundamental role of aldosterone and vascular MR in the regulation of arterial tone, vascular contractility, and cell proliferation. However, the intricate transduction machinery activated by vascular MRs has begun to be revealed with the help of transgenic rodent models and novel transcriptional analysis approaches. Specifically, in this chapter, we review and discuss the most recent contributions about the fine-tuning that the MR exerts on the expression and function of ion channels that participate in calcium handling of vascular cells and the therapeutic implications for hypertension and cardiovascular diseases.

Keywords: calcium channels, calcium handling, vascular smooth muscle, aldosterone, mineralocorticoid receptor

1. Introduction

Recent research efforts on the mineralocorticoid receptor (MR) signaling have revealed a cluster of new pathophysiological mechanism mediated by vascular MR in which aldosterone (Aldo) plays a pivotal role; however, the molecular pathways are not completely elucidated. In this chapter, we review and discuss novel contributions about the structure, ligand activation, and additional mechanisms that confer selectivity for Aldo of the MR in vascular tissues. In addition, we review the fine-tuning that the MR exerts on the expression and function of

ion channels that participate in Ca^{2+} handling of vascular smooth muscle cells and the therapeutic implications for hypertension and cardiovascular diseases.

In 1972, Crabbé demonstrated that Aldo was interacting with cytoplasmic receptors and that the steroid-protein complex acted as activator triggering the synthesis of mRNA and proteins [1]; thus, Aldo was the first identified mammalian steroid hormone that exerted transcriptional actions via some kind of cytoplasmic/nuclear receptors [2]. Several classes of mineralocorticoid receptors were identified in both epithelial and surprisingly in non-epithelial tissues such as cardiomyocytes, endothelial cells (ECs), and vascular smooth muscle cells (VSMCs) [2], auguring the future actions of Aldo in the cardiovascular system.

2. Mineralocorticoid receptor structure and expression in vascular tissues

The MR is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily [3]. MR was originated by a process of gene duplication from a common ancestor that diverged into the glucocorticoid receptor (GR) and the MR [4]. In 1987 the group of Arriza et al. cloned the MR from human placenta [5]. The human MR (hMR) is coded by a unique gene (*Nr3C2*, due to its belonging to the subfamily 3 of nuclear receptors, group C, member 2) located in chromosome 4, locus q31.1, and with a length of about 75Kb. The gene encodes a polypeptide chain of 984 aa (~107 KDa) [5]. The orthologous gene in rat encodes for a protein of 981 aa (**Figure 1**) and share an identity of 90.1% with the hMR. *Nr3C2* contains 10 exons; the first 2 of them (1α and 1β) comprise the 5′-noncoding sequences, whereas the following exons (2–9) are harboring the functional domains of the protein. It has been reported that at

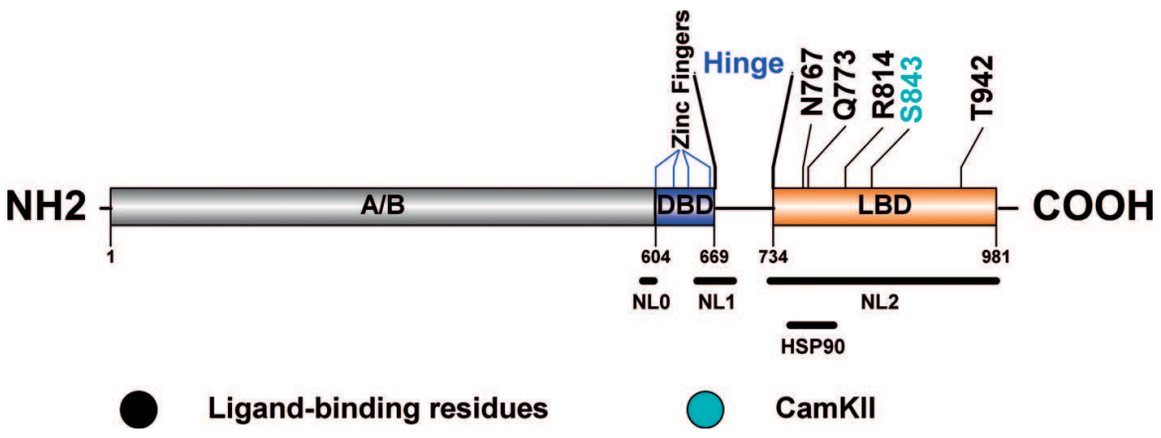

Figure 1. Mineralocorticoid receptor structure. Linear representation of rat MR sequence with respective protein domains. The MR contains an N-terminal transactivation domain of variable lengths (A/B) and a DNA binding domain (DBD) with two zinc fingers involved in the recognition of specific DNA sequences within the promoters of target genes and named hormone response elements (HRE); a flexible hinge is connecting the DBD to the ligand-binding domain (LBD) in the C-terminal region. The residues N767, Q773, R814, and T942 are part of the ligand-binding pocket. The MR also contains three nuclear localization signals (NL0, NL1, and NL2) and multiple phosphorylation sites, and between them is Ser843 that is a target of calcium/calmodulin-dependent protein kinase type II (CamKII).

least three variants of MR mRNA (α, β, and γ) are encoded in a tissue-specific manner under the control of different gene promoters [6].

The MR receptor has the same protein structure as other members of the nuclear receptor superfamily. The MR is composed of an A/B domain (1–604 aa) with a transactivation function and several serine and threonine phosphorylation sites [7] and a DNA binding domain (DBD, 604–699 aa) with two zinc finger motifs that recognize DNA-specific sequences named hormone response elements (HRE), normally found in the promoters of target genes [8]. After the DBD, the hinge D region (670–733 aa) is found and, finally, the C-terminal region (734–981 aa) that is harboring the ligand-binding domain (LBD) with a pocket (which comprises Asp767, Gln773, Arg814, and Thr942) involved in the recognition of agonist and antagonist [9] (**Figure 1**).

2.1. Mineralocorticoid receptor expression in different tissues

The direct and specific actions of Aldo require MR expression in target tissues. For a long time, it was thought that MR was expressed exclusively in kidney epithelial cells and that Aldo was secreted only by the adrenal gland. However, a cumulative evidence has showed the presence of MR in non-epithelial tissues, such as the colon, salivary glands, trachea, heart [10], adipocytes [11], brain [5], skeletal muscle [12], leucocytes, macrophages [13], and vessels [14–19] (**Table 1**).

Tissue or cell type	Detection method	Reference
Kidney	NB	[5]
Gut (gastrointestinal tract)	NB	[20]
Brain	NB	[5]
Hippocampus	NB	[5]
Heart	Im	[10]
Skeletal muscle	WB	[12]
Endothelial cells	RT-PCR	[17]
Vascular tissues		
Aorta	Im, WB, [^{3}H]Aldo bindings	[10, 16, 19, 21, 22]
Carotid artery	Im	[10]
Cerebral artery	WB	[18]
Coronary artery	RT-PCR, Im, WB	[10, 16, 19]
Humeral artery	Im	[10]
Mesenteric artery	Im, RT-PCR, WB	[10, 15, 19]
Pulmonary artery	Im, NB	[10, 14]
Renal artery	Im	[10]
Saphenous vein	RT-PCR, WB, WB	[23]

Tissue or cell type	Detection method	Reference
Umbilical vein		[23]
Adipocytes	Im	[11]
Macrophages	PCR, Im	[13]
Lymphocytes	PCR	[13]

Im, immunohistochemistry; NB, Northern blot; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; WB, Western blot technique.

Table 1. Cell types and tissues expressing MR.

Specifically in blood vessels, the group of Marc Lombès showed for the first time the expression of MR in ECs and SMCs of the aorta and pulmonary artery by immunostainings and [^{3}H]Aldo binding [10]. Although with a low level of immunostaining signal, the MR was found in small arteries such as the carotid, humeral, mesenteric, coronary, and renal arteries. Interestingly, neither immunostaining nor [^{3}H]Aldo binding allowed the detection of MR in the vena cava and portal vein [10]. Hatakeyama et al. detected MR mRNA in primary cultures of ECs and SMCs from human pulmonary arteries [14]. Later, Takeda et al. demonstrated the presence of MR mRNA in mesenteric arteries of stroke-prone spontaneously hypertensive rats (SHRSP) [15]. Using RT-PCR the MR mRNA was detected in a human aorta [21]. Jaffe and Mendelsohn also demonstrated that MR was expressed in VSMCs from aorta and heart vasculature, specifically in coronary arteries [16]. In the case of cerebral arteries (CA), MR expression has been showed by Western blots, where MR levels were higher in CA from females than males [18]. All of these data established the foundation for understanding MR action in vascular tissues. Nowadays we know that MR is indeed expressed in the cardiovascular system supporting its direct role in vascular pathophysiology.

3. Mineralocorticoid receptor: mechanism of activation and regulation in vascular tissues

In basal conditions, the MR is located at the cytoplasm forming a complex with heat-shock proteins, Hsp90 and Hsp70, that stabilize its structure in a conformation where the ligand-binding site is ready to interact with the hormone [24, 25]. Once the ligand is bound, the MR is subjected to a series of conformational changes (**Figure 2**). The interaction between N-terminal and C-terminal domains of MR induces the dissociation of heat-shock proteins and corepressors, allowing MR translocation to the nucleus. However, it has been reported also that Hsp90 follows MR into the nucleus [26]. Inside the nucleus the MR dimerizes, binds to HRE, and recruits a co-regulator complex to induce the transcription of target genes [27, 28]. Interestingly, in some animal models of kidney and cardiac injury that showed normal plasma Aldo concentrations, the MR is activated through a ligand-independent pathway, involving a direct interaction between a small GTPase, Ras-related C3 botulinum toxin substrate 1 (Rac1) and the MR [29], though evidence of this pathway is still lacking in vessels.

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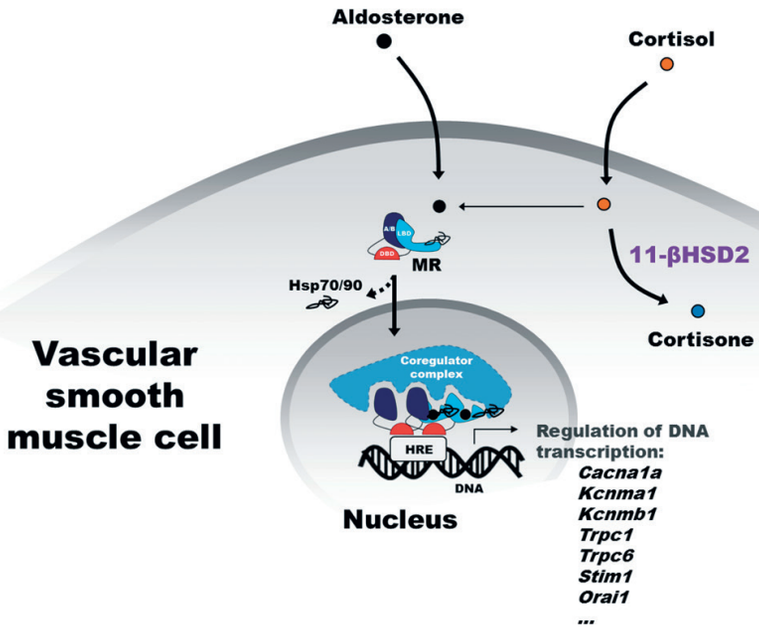

Figure 2. Schematic representation of the MR activation mechanism by Aldo in vascular smooth muscle cells. Once MR binds Aldo in the cytoplasm, it is subjected to conformational changes that allow the dissociation of heat-shock proteins (Hsp70/90), the unmasking of nuclear localization signal, and finally the MR translocation to the nucleus where the MR dimerizes (homodimer complex) and binds to hormone response elements (HRE). The MR dimer recruits a co-regulator complex for regulating the transcription of target genes. Both Aldo and cortisol bind to MR with similar affinity. The mechanism that confers MR selectivity for Aldo depends on the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11 βHSD2), which converts cortisol to cortisone, the latter has a low affinity for MR. Some of the MR target genes in SMC from different vascular beds are listed [19, 30–32]. Abbreviations: *Cacna1a*, VDCC subunit alpha1 A; *Kcnma1*, BK_{Ca} channel (alpha subunit); *Kcnmb1*, BK_{Ca} channel (beta subunit 1); *Trpc1*, transient receptor potential cation channel subfamily c member 1; *Trpc6*, transient receptor potential cation channel subfamily c member 6; *Stim1*, stromal interaction molecule 1; *Orai1*, ORAI calcium release-activated calcium modulator 1.

The high homology between the LBD structure of MR and GR receptors helps to explain why the MR binds cortisol and corticosterone and glucocorticoids with similar affinity with Aldo [33]. Cortisol and Aldo bind to hMR with similar affinity [5, 9]. Because the serum levels of cortisol are higher (from 100 to 1000 times more) than Aldo, then it is expected that the occupancy of MR by cortisol predominates. However, a mechanism that confers MR selectivity for Aldo depends on the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), which converts cortisol to cortisone, the latter has a low affinity for MR [34]. Several papers have demonstrated the co-expression of MR with a functional 11βHSD2 in different vascular beds including mesenteric [15] and coronary arteries [16, 35], though with an indirect determination in CA [36]. These studies support that vascular tissues are Aldo-specific targets and that the action of 11βHSD2 is the physiological mechanism that excludes the activation of MR by cortisol. Furthermore, these data support that the MR-Ado complex has greater stability and higher transcriptional efficiency than the MR-cortisol complex [9].

3.1. Importation of MR into the nucleus

Immunostainings of MR in SMC from coronary arteries have showed the presence of MR in both cytoplasm and nucleus, even in the absence of a ligand. After exposure to Aldo, MR

is located mainly in the nucleus [16]. Like other nuclear receptors, the MR is a protein of a considerable molecular mass (~107 KDa) that exceeds the calculated size for its passive diffusion through the nucleus; thus, it requires specific signals for its nuclear transport. Specifically the importation of MR into the nucleus is controlled through three nuclear localization signals (NLS): the first NLS (NL0) is a serine-/threonine-rich sequence located at the N-terminal region, the second is a NLS (NL1) located at the DBD, and the third is a NLS (NL2) within the LBD. The presence of several NLS in different regions of the MR structure suggests a redundant mechanism to assure its mobilization toward the nucleus as part of the essential mechanism of its transcriptional activity [37].

3.2. MR posttranslational modifications

The MR is subjected to several posttranslational modifications (PTM) such as phosphorylation, ubiquitination, sumoylation, and acetylation that regulate its localization, activity, and stability. Phosphorylation is the most common PTM since the MR contains more than 30 (putative and experimentally assessed) phosphorylation sites that allow to consider it as a phosphoprotein (**Figure 2**). The multiple phosphorylation sites of the MR generate a double band in SDS-PAGE due to a shift of approximately 30 kDa in its apparent molecular mass. The physiological function of all these phosphorylation sites is still under study or completely unknown as in the case of vascular MR. A report has shown that the phosphorylation of Ser843 (in the LBD) prevents MR ligand binding and activation [38]. In contrast, Amazit et al. reported an increase in the MR phosphorylation after Aldo binding, suggesting a ligand-dependent process [39]. Walther et al. reported that phosphorylation of residues inside the NL0 might modulate the MR transport into the nucleus [37]. Finally, Faresse et al. found that the MR is monoubiquitinylated at its basal state and that the Aldo-stimulated MR phosphorylation induces its polyubiquitinylation, destabilization, and degradation [40]. The physiological role of MR-PTM has not been determined in VSMCs.

3.3. Genomic effects of aldosterone in vessels

Aldo exerts its effects in vascular tissues via non-genomic (which are not subjects of this chapter) and genomic MR-dependent pathways. In the case of MR-dependent genomic actions in VSMCs, Jaffe and Mendelsohn investigated the MR-mediated gene transcription activity in SMC of human coronary arteries (HCSMCs) by microarray and quantitative RT-PCR assays. These researchers showed that Aldo modulated the expression of VSMC genes that contribute to vascular inflammation and fibrosis. Additionally, by using a MR response element (MRE) reporter driving the expression of the luciferase gene, they demonstrated that Aldo activated MR in HCSMCs. The Aldo transcriptional effects were regulated in a dose-dependent manner, starting at 1 nM, which is consistent with the Kd for Aldo-MR interaction of ~1–2 nM [16]. Similarly, Newfell et al. evaluated the gene expression profile in Aldo-treated aorta ex vivo, identifying 72 genes that were regulated by Aldo, some of them in a concentration-dependent fashion (1, 10, 30, and 100 nM). Between the Aldo-regulated genes, several of them are involved in oxidative stress, nitric oxide (NO) signaling, vascular proliferation, and fibrosis. Moreover, it has been showed that the MR transcriptional activity is blunted by MR antagonists, and

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actinomycin D (a transcriptional inhibitor) supporting an MR-dependent effect of Aldo in vessels [16, 19, 30, 41]. Furthermore, an increasing body of evidence has underlined the ability of MR to modulate the expression of ion channels in several vascular beds, unveiling the role of MR in vascular physiology and pathology [19, 30, 42, 43].

4. Pathological role of vascular mineralocorticoid receptor in blood vessels

It has been confirmed that MR presents extrarenal actions [42, 44, 45] as Na^+ handling alone cannot fully explain the development of hypertension and associated cardiovascular mortality; but these actions are still poorly understood. In fact, there are multiple clinical studies in which mineralocorticoid receptor antagonists (MRA) reduce the incidence of heart attacks and cardiovascular mortality [46, 47].

4.1. Role of vascular MR in oxidative stress

In vitro and in vivo data suggest a vascular MR activation in stimulating oxidative stress, inhibiting vascular relaxation, and contributing to vessel inflammation, fibrosis, and remodeling. MR activation may promote vascular aging and atherosclerosis contributing to the pathophysiology of heart attack, stroke, and possibly hypertension [42]. The balance between damaging reactive oxygen species (ROS) and protective NO determinates the vascular oxidative stress. ROS interact with NO decreasing the NO bioavailability. In vivo experiments in rats support that activation of MR signaling contributes to the vascular dysfunction induced by βAR overstimulation associated with endothelial NO synthase uncoupling reducing NO production and bioavailability [48]. Meanwhile, in the presence of endothelial dysfunction, vascular injury, or high vascular oxidative stress (for instance, in patients with cardiovascular risk factors), ROS production increases via VSMC-MR-mediated activation of NADPH oxidase (a ROS generator) [23, 49, 50] promoting impaired EC-dependent vasorelaxation and consequently increasing vasoconstriction and blood pressure (BP) [51].

4.2. Role of MR in vascular remodeling

Vessel injury induces a pathological response termed vascular remodeling which contributes to human ischemic vascular disease. Adverse vascular remodeling limits vessel lumen diameter and increases vascular stiffness associated with fibrosis, thereby contributing to organ ischemia and hypertension. MR activation contributes to vascular remodeling by acting synergistically with endothelial damage, angiotensin II (Ang II), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) [52–55]. These processes involve both genomic (upregulation of genes involved in cell migration, proliferation, and matrix modulation) and non-genomic mechanisms (via MAPK and the c-Src/Rho) [56]. In the pulmonary artery, MR activation induced the proliferation of VSMC, an effect prevented by spironolactone [57]. Moreover, in a VSMC-MR knockout mouse model, carotid injury-induced and

aldosterone-enhanced vascular fibroses were attenuated; thus, VSMC-MR is necessary for aldosterone-induced vascular remodeling [58]. In aged VSMC-MR-deficient mice (18-month-old), a decrease in aortic collagen content was found [42], suggesting that VSMC-MR play a role in vascular fibrosis. Unlike in an aldosterone/salt hypertension model, the specific VSMC-MR inactivation also leads to the attenuation of arterial stiffening preventing the cell-matrix attachment proteins but without significant modification in vascular collagen/elastin ratio [22]. Other studies support that, after injury, aldosterone-infused animal developed vascular remodeling and MR antagonist reversed those effects [59–61]. Pharmacological inhibition of MR has also demonstrated beneficial effects such as increased lumen and outer diameters of the middle cerebral artery of spontaneously hypertensive stroke-prone rats [62]. Moreover, in a clinical study, treatment with the MR antagonist eplerenone improves the degree of arterial stiffness in hypertensive patients [63]. In conclusion, these studies support that VSMC-MR plays a direct role in vascular remodeling.

4.3. Participation of MR in vascular inflammation

MR plays a key role in the pathogenesis of vascular disease including atherosclerosis and hypertensive vasculopathy, where the role of inflammation has been studied in the last years. In patients with atherosclerosis, high levels of aldosterone in serum predict a substantial increase in subsequent myocardial infarction or death. In vitro studies with human VSMC and ECs have shown that MR activation directly promotes the expression of inflammatory genes [64]. Interestingly, in an in vivo model, spironolactone reduced the number of inflammatory cells in the grafted vein without changing total SMC content, suggesting that MR signaling may contribute to graft remodeling through inflammatory processes rather than SMC hypertrophy [65]. Moreover, in experimental models of atherosclerosis, it was confirmed that the plaque progression was enhanced by Aldo and prevented by MR antagonists [66–68]. The pro-atherogenic genes (*CTGF*, *MT1*, and *PGF*) are also vascular MR-regulated genes [41]. MR signaling also contributes to vascular inflammation in animal models of hypertension. In experimental models of hypertension, MR inhibition reduced the vascular inflammation even without changes in BP, supporting that MR activation participates in vascular inflammation and damage through a BP-independent process [69]. Vascular calcification is a late stage found in atherosclerosis, particularly in the elderly and in patients with renal failure [70]. In VSMCs from human coronary artery, MR activation by Aldo upregulated the expression of genes implicated in vascular calcification, including bone morphogenetic protein 2 (*BMP-2*), alkaline phosphatase (*ALP*), and osteoprotegerin [16]. Also, in an in vitro model, MR activation by Aldo or cortisol stimulated vascular *ALP* [71]. MR is also involved in vascular calcification by regulating the expression of the phosphate transporter *Pit1*, which has an osteogenic function in the smooth muscle ameliorated by spironolactone [72]. However, in a different in vitro study using VSMC isolated from the aorta, it was showed that pro-calcification effects of corticosterone and 11-DHC are mediated directly by MR, but the expression of *Osterix*, *BMP-2*, and *Pit-1* was unaltered [73]. These in vitro studies support that MR is involved in the late stage of atherosclerosis: vascular calcification. All these studies support that vascular MR activation participates in the inflammatory response and contributes to the complications associated with atherosclerotic vascular disease.

4.4. Vascular MR and hypertension

Hypertension represents an aging-associated cardiovascular risk factor. It is known that renal MR regulates the BP and the MR has been considered an antihypertensive target for decades. The association between high levels of Aldo and hypertension was proposed when some forms of hypertension were found associated to primary hyperaldosteronism; also, the positive correlation of high levels of Aldo with high MR expression and hypertension has pointed out to a key role of MR in the establishment of the hypertensive phenotype. Moreover, about 50 years ago, the MR antagonist spironolactone decreased BP in hypertensive patients [74] and in patients with other types of hypertension [75]. The antihypertensive effects of MRA have been analyzed in clinical trials demonstrating a BP reduction in hypertensive patients with primary aldosteronism [76–78] with no changes in plasma K^+ concentration, a marker of renal MR activation [44, 79]. The meta-analysis by Dahal et al. showed that spironolactone reduced systolic BP and this effect was not associated with an increased risk of hyperkalemia compared to placebo [80]. Antihypertensive effects of MRA were analyzed also in resistant hypertension that affects at least 10–15% of all patients. Several studies support that low-dose spironolactone provides significant additive BP reduction in subjects with resistant hypertension [75, 81–85]. PATHWAY-2 was the first randomized and controlled trial to compare spironolactone with other BP-lowering drugs in a well-characterized population of patients with resistant hypertension. In this study it was demonstrated that MRA reduced systolic BP with no hyperkalemia risk [86]. In addition, a role of MR in pulmonary hypertension has been recently identified. MRA treatment initiated at the time of the pulmonary arterial hypertension stimulus prevents the pulmonary vascular hyperplasia and reduces systemic BP [57, 87]. Thus, MR activation may be equally important in patients with and without an established diagnosis of primary aldosteronism. The pathogenesis of MR-associated hypertension in the presence of physiological levels of Aldo in plasma might be mediated by MR activation by other pathways, for instance, MR overexpression, sensitivity, and/or overstimulation by other factors.

The effect of MR blockade in the development of hypertension has been also assessed in experimental models. In the Dahl salt-sensitive model, MRA attenuated the progressive rise in systolic BP in rats fed with a high-salt diet [88, 89]. Whole body disruption of MR in mice results in neonatal lethality from dehydration by renal Na^+ and water loss; thus, transgenic mouse models allowing cell-specific targeting of MR expression have been used to understand the role of MR in vascular tissues and its potential implication in BP regulation. The participation of vascular MR in BP regulation has been analyzed by using two different mouse models: a tamoxifen-inducible VSMC-specific MR inactivation model (via the smooth muscle actin promoter [42]) and a constitutive model of VSMC-specific MR inactivation (via the SM22 promoter; [22]). No transgenic models with targeted MR overexpression in the VSMCs have been reported yet.

The genetic inactivation of MR in adult (2 months of age) mice prevented the increase in BP induced by aging. SMC-MR-deficient mice developed reduced spontaneous myogenic tone. However, the vascular structure and stiffness of resistance arteries from aged SMC-MR-deficient were similar to those from control mice, supporting the notion that SMC-MR contributes to vascular tone and BP regulation independently of structural changes in the

vasculature [42]. The constitutive model of VSMC-specific MR inactivation reported a similar basal BP decrease in 5-month-old MR-KO mice [22]. The BP phenotype in both inactivated VSMC-MR model mice is independent of Na^+ intake and renal MR function supporting a role for VSMC-MR in BP regulation. Interestingly, tamoxifen-inducible VSMC-MR inactivation prevented the in vivo increase in BP induced by Ang II infusion but not by aldosterone-salt challenge [22, 42]. Inactivation of VSMC-MR was also shown to decrease the contractile response to KCl and extracellular Ca^{2+} [62]. The role of the vascular MR could also depend on the vascular bed that is considered. In the future, the use of transgenic models will allow us to decipher the contribution of endothelial MR and VSMC-MR in the different vascular beds and the possible implication in BP regulation [90].

5. Calcium handling proteins are targets of vascular MR receptors

5.1. L-Type Ca^{2+} channel

Recognized as a universal second messenger in various cellular processes and cell types, Ca^{2+} signal plays a critical role in many cellular processes, including, but not limited to, gene transcription and excitation-contraction (EC) coupling [91, 92]. Although almost all biological responses are mediated by Ca^{2+}-dependent and Ca^{2+}-controlled processes, Ca^{2+} signals need to be finely coordinated and precisely regulated. Ubiquitously expressed in the whole body, $Ca_v1.2$ is the main route of Ca^{2+} entry in VSMCs, essential for vascular EC coupling and control of myogenic tone [93]. As a heteromultimeric channel, L-type Ca^{2+} channel (LTTC) is formed by four associated subunits: $Ca_v1.2\alpha1C$, $Ca_v1.2\beta$, $Ca_v1.2\alpha2\delta$, and $Ca_v1.2\gamma$. Undebatable the main subunit, the pore-forming $Ca_v1.2\alpha1C$ region, has also been the target of drugs with antihypertensive properties [94–96], although its effectiveness has been achieved only in a subset of hypertensive patients [97]. Importantly $Ca_v1.2\alpha1C$ channels are expressed as two distinct tissue-specific transcripts of *Cacna1c* driven by two alternative promoters P1 and P2, encoding, respectively, for a long "cardiac" ($Ca_v1.2$-LNT) and for a short "vascular/brain" ($Ca_v1.2$-SNT) N-terminal region [98]. In VSMCs, LTTC is activated in response to the membrane depolarization, allowing a small fraction of Ca^{2+} influx, which is sufficient to trigger VSMC contraction. Thus, sustained voltage-dependent Ca^{2+} influx through the LTCCs maintains a tonic level of vasoconstriction and provides an excitatory template upon which endogenous vasoactive substances may act to modulate arterial diameter and BP.

Although previous studies have demonstrated that aldosterone modulates VSMC Ca^{2+} currents [99–101], the mechanisms remain to be determined. A landmark study showed that VSMC-specific MR knockout mice (VSMC-MR-KO) are protected against the age-associated rise of BP [42]. Importantly, aged VSMC-MR-KO mice showed decreased myogenic tone and attenuated vascular contraction in mesenteric arteries in response to a LTCC opener. Moreover, mRNA level of $Ca_v1.2\alpha1C$ subunit was dramatically downregulated in aortas from aged VSMC-MR-KO mice, suggesting that MR may regulate VSMC $Ca_v1.2$ expression. However, this phenomenon seems indeed to be an age-dependent effect, since a latter study did not validate, at protein level, the downregulation of $Ca_v1.2$ in aortas from young

VSMC-MR-KO mice [102]. Furthermore, during the aging process, MR expression increases in resistance vessels along with a decline in the microRNA (miR)-155 abundance, suggesting that $Ca_v1.2$ is a downstream target of miR-155 regulation [103].

Adding more pieces to the puzzle, we recently showed in cardiomyocytes that aldosterone regulates $Ca_v1.2$-LNT by recruiting MR onto targeted genomic regions in "cardiac" *Cacna1c* P1-promoter [19]. Importantly, we deciphered that aldosterone, through MR-dependent mechanism, dramatically activates the "cardiac"-specific *Cacna1c* P1-promoter, even in blood vessels, conferring a new molecular signature to $Ca_v1.2\alpha1C$ in this tissue that minimizes Ca^{2+} channel blocker actions, a mechanism that might participate to treatment-resistant hypertension, as recently proposed [86]. These findings were further validated using a hypertensive rat aldosterone-salt model, as previously described [104]. Although our data showed that aldosterone/MR impairs 1,4-dihydropyridine sensitivity in VSMC through alternative splicing of $Ca_v1.2\alpha1C$, further studies are needed to validate whether this mechanism participates in the resistant hypertension.

5.2. Ca^{2+}-activated potassium channels

Ca^{2+}-activated potassium channels (KCa), mainly the large conductance KCa channels (BKCa), have been recognized as another important target of MR in blood vessels [105]. BKCa plays a critical role in limiting arterial contraction by producing VSMC hyperpolarization through transient outward K^+ current in response to increased intracellular Ca^{2+} concentration [106]. However, three subtypes of KCa have been identified in blood vessels and categorized according to their conductance: small (SKCa), intermediate, and BKCa. Small- and intermediate-conductance channels are mainly expressed in the ECs, while BKCa channels are predominately expressed in VSMCs.

Previous studies have shown that increased plasma aldosterone concentration enhances vascular KCa function [105]. Oppositely, it was demonstrated that mice lacking the pore-forming BKCaα subunit led to an elevation of BP resulting from hyperaldosteronism, which was accompanied by decreased serum K^+ levels, as well as increased vascular tone in small arteries [107]. Accordingly, impaired acetylcholine-mediated relaxation in isolated coronary arteries has been shown in mice model with cardiac-specific overexpression of aldosterone synthase (MAS mice) [30]. These findings correlate with decreased mRNA and protein expression of BKCa α and $\beta1$ subunits in the heart and coronary artery of MAS mice. Moreover, in vitro treatment of rat aortic VSMCs with increasing concentrations of aldosterone led to a reduced BKCa subunit expression in a concentration-dependent manner. Thus, these findings suggest that augmented local aldosterone production likely acts in a paracrine fashion way suppressing BKCa expression in the surrounding coronary VSMC, thereby contributing to the impaired endothelium-dependent VSMC relaxation. Intriguingly, despite aged VSMC-MR-KO mice displaying lower BP than age-matched WT mice, no significant changes were observed in aortic mRNA expression and function of BKCa in mesenteric VSMC [42]. Furthermore, aldosterone-treated aorta for 24 h with 10^{-8} M of aldosterone did not modify mRNA expression of BKCa α and $\beta1$ subunits [19]; thus, further studies are needed to clarify the effect of MR activation in the expression and activity of BKCa channels.

As mentioned above, SKCa is predominantly expressed in ECs, where it contributes to endothelium-derived hyperpolarization (EDH) of VSMC resulting in vasorelaxation of resistance arteries [108]. In a previous study, circulating aldosterone level was significantly higher in mice fed with high-fat diet (HFD) compared to lean mice; however, despite the restoration of endothelium-dependent vasodilation, eplerenone treatment further increased plasma aldosterone levels of HFD-fed obese mice [109]. Recently, using similar obese model, plasmatic aldosterone concentration was also augmented in male and female mice, whereas no change was found in endothelial cell-specific MR knockout mice (EC-MR-KO) subjected to HFD [110]. In males, obesity impaired NO-dependent vasodilation of resistance arteries, which was compensated by enhancement of EDH of VSMC along with an increase in mesenteric protein expression of SKCa3, while any change was observed in EC-MR-KO. On the other hand, in females, EDH component of VSMC relaxation was impaired, whereas the expression of SKCa3 remained unchanged in control and EC-MR-KO underwent to HFD [110]. Altogether, these results uncover distinct sex-specific mechanisms driving vascular dysfunction, suggesting personalized therapies to prevent vascular disorders.

5.3. Transient receptor potential channels

In VSMC, Ca^{2+} entry from the extracellular space involves a variety of plasmalemmal Ca^{2+} channels, which also involve the superfamily of transient receptor potential (TRP) channels, such as TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), and TRPP (polycystin) [111]. Widely expressed in visceral and vascular SMC, changes in the expression and activity of these channels are implicated in a variety of physiological and pathophysiological consequences [112]. Although TRPM subfamily contains eight isoforms (TRPM1–8), which exhibit a variety of cation permeability, only TRPM6 and M7 seems to be Ca^{2+} and Mg^{2+} permeable. Interesting, aldosterone (100 nM) transiently upregulates mRNA TRPM7 expression in rat VSMC from 2 to 6 h after the onset of treatment, restoring to control level after 24 h of treatment [113]. However, up to date, no studies have been done to evaluate whether aldosterone modulates the expression of TRPV and TRPP channels.

Originally thought to contribute solely to restoring Ca^{2+} concentration under store depletion, creating a capacitative Ca^{2+} entry, commonly termed as store-operated Ca^{2+} entry (SOCE), the role of SOCE is much more diverse than just refilling Ca^{2+} stores [114] but also contributing to vascular contractility, VSMC proliferation, and differentiation [112]. In addition, Ca^{2+} entry from the extracellular space may also occur through Ca^{2+}-permeable store-independent channels, named as receptor-operated channels (ROC), which their activity depends on second messengers produced by downstream effectors from a vast array of G protein-coupled receptors [114]. TRPC subfamily comprises seven members (TRPC1–TRPC7), with the TRPC2 gene being a pseudogene in humans [114].

Although TRPC1 seems to be the most abundant isoform expressed in rat mesenteric arteries, only the expression of TRPC6 was increased in deoxycorticosterone acetate (DOCA)-salt hypertensive rats [31]. Moreover, A7r5 cells treated with aldosterone (1 μM for 24 h) also displayed increased mRNA and protein levels of TRPC6 [31]. Accordingly, it was shown that coronary rings cultured for 7 days with aldosterone (100 nM), without fetal bovine

serum to preserve the contractile phenotype, displayed higher coronary contractility in both endothelium-denuded and endothelium-intact rings, while co-treatment with spironolactone prevented this effect [115]. Recently, we demonstrated that rat aorta treated with aldosterone (10 nM for 24 h) did not reveal changes in the expression of TRPC1, C3, C4, C5, and C6 [19]. Altogether, these studies suggest a concentration-dependent increase of TRPC channels, since we have previously demonstrated an upregulation of TRPC1, C4, and C5 in cardiomyocytes upon aldosterone concentrations higher than 100 nM [43]. Moreover, one of the features of metabolic syndrome is the elevated plasma aldosterone level [116], which has been associated with increased TRPC1 and TRPC6 expression in coronary arteries compared to lean pigs [32].

Another critical component of SOCE is the protein Orai (comprising Orai1, Orai2, and Orai3), which forms a family of highly Ca^{2+}-selective channels that are regulated by stromal-interacting molecules (STIM1 and STIM2) [111, 112]. In neonatal cardiomyocytes, Orai1 was significantly increased by 100 nM and 1 μM aldosterone treatment, whereas lower concentrations (1 and 10 nM) had no effect [43]. However, Stim1 expression remained unchanged even at the highest concentration tested (1 μM) [43]. Similarly, we recently observed, in blood vessels, that treatment with 10 nM of aldosterone for 24 h does not affect the expression of either Orai1 or Stim1 [19].

6. Perspectives

Hypertension is a substantial public health problem, affecting 25% of the adult population in industrialized societies. This disorder is a major risk factor for many common causes of morbidity and mortality including stroke, myocardial infarction, congestive heart failure, and end-stage renal disease. Thus, substantial effort has been devoted to defining the pathogenesis of BP variation. Aldo, through the activation of MR in tubular epithelial cells, has a well-known function on water balance and BP homeostasis. The renal hemodynamic consequences of excess mineralocorticoids—Na^+ and water retention and K^+ secretion—ultimately lead to hypertension. However, the kidney is no longer regarded as the primary site for mineralocorticoid modulation of BP. MR is consistently expressed in both ECs and VSMCs of blood vessels, and its activation by Aldo at pathological concentrations (10 nM) is associated with several types of vascular dysfunction, including atherosclerosis and hypertension. However, despite the recent understanding about the mechanisms involved in the activation of MR mainly in pathological conditions, further research is still required to determine the physiological role of MR-VSMCs in blood vessels.

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Conflict of interest

The authors declare no conflict of interest.

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