Regulation and function of endothelial cell mineralocorticoid receptor in cardiovascular physiology and pathology

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Abstract

The steroid hormone aldosterone activates the mineralocorticoid receptor (MR), a steroid receptor transcription factor that contributes to cardiovascular disease. While MR is best known for its role in enhancing sodium and water reabsorption in the renal epithelia to increase blood volume, clinical trials have demonstrated benefit for MR antagonism disproportionate to its antihypertensive effect. Thus antagonism of non-renal MR likely contributes to clinical benefits of these drugs. Endothelial cells compose the innermost layer of the blood vessel and participate in regulation of vasomotor function in health and disease. MR is expressed in endothelial cells, and the literature suggests a role for MR in exacerbating vascular pathology in the presence of numerous cardiovascular risk factors. Estrogen receptor α (ERα), another transcription factor that is activated by estrogen, is also expressed in endothelial cells. Endothelial ERα protects against cardiovascular disease, correlating with the epidemiologic finding that premenopausal women have less cardiovascular disease than age-matched men. We tested the hypothesis that one mechanism for sex differences in cardiovascular disease is that estrogen-activated ERα interferes with the transcriptional effects of aldosterone-activated MR in ECs. First we demonstrated that in HEK293 cells in which we expressed MR and an MRE-luciferase reporter, cotransfection of ERα blocked MR-mediated transcription. The same effect of estrogen- and ERα-mediated inhibition of MR-activated transcription was seen in EAHy926 endothelial cells transfected with the MRE-luciferase reporter. Furthermore, aldosterone-stimulated upregulation of endothelial mRNA and protein of the pro-inflammatory adhesion molecule ICAM-1 was attenuated with coadministration of estrogen. An in vitro leukocyte adhesion assay in which aldosterone is known to promote pro-inflammatory leukocyte adhesion to endothelial cells was likewise inhibited by estrogen administration, suggesting physiologic relevance of ERα-mediated inhibition of MR transcriptional function. Hence in the vascular endothelium, estrogen-activated ERα may achieve its cardioprotective effects in part through inhibition of the harmful effects of aldosterone-stimulated MR. This finding may create novel therapeutic opportunities for targeting the role of endothelial cells in cardiovascular disease.

To further investigate the role of endothelial MR in vascular function and disease in vivo, we created a mouse with MR specifically deleted from EC (EC-MR-KO) but with intact leukocyte MR expression and

normal renal MR function. Although endothelial cells (EC) express MR, the contribution of EC-MR to blood pressure and resistance vessel function remains unclear. Telemetric blood pressure studies revealed no difference between male EC-MR-KO mice and MR-intact littermate controls in systolic, diastolic, circadian, or salt-sensitive blood pressure or in hypertensive responses to aldosterone/salt or angiotensin II. Vessel myography demonstrated normal vasorelaxation in mesenteric and coronary arterioles from EC-MR-KO mice. After exposure to angiotensin II-induced hypertension, endothelial-dependent relaxation was impaired in coronary and mesenteric arterioles from MR-intact mice. This impairment was prevented specifically in EC-MR-KO mesenteric vessels, which showed increased maximum responsiveness to Ach, compared to MR-intact vessels. These data support that EC-MR plays a role in regulating endothelial function in hypertension. Although there was no effect of EC-MR deletion on mesenteric vasoconstriction, EC-MR-KO coronary arterioles showed decreased constriction to endothelin-1 and thromboxane agonist at baseline and after exposure to hypertension. These data support that EC-MR participates in regulation of vasomotor function in a vascular bed-specific manner that is modulated by risk factors such as hypertension.

In addition the literature supports a role for MR in enhancing atherosclerosis. Atherosclerosis is an inflammatory disorder of the vasculature that results in heart attacks and strokes. Healthy mice infused with two weeks of aldosterone show no increase in leukocyte infiltration of the blood vessel, but aldosterone treatment in mice exposed to high fat diet and hyperlipidemia significantly augments leukocyte infiltration, suggesting that MR activation, specifically in the presence of such cardiovascular risk factors, enhances the vascular inflammation characteristic of atherosclerosis. To clarify the role of endothelial MR in atherosclerosis, EC-MR-KO mice were crossed with ApoE-/- mice to generate atherogenic EC-MR-KOApoE-/- mice and MR-intact/ApoE-/- littermates. Mice were fed high fat diet for four weeks and then the aorta was collected and immunohistochemistry was used to assess plaque size and lipid content. A trend towards decreased plaque size in EC-MR-KO/ApoE-/- mice compared to MR-intact/ApoE-/- mice was observed, suggesting endothelial MR may play a role in enhancing plaque development. Future studies will be needed to confirm this finding and to explore additional mechanisms by which EC MR contributes to atherosclerosis.

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Table of Contents

List of Tables

List of Figures

Abbreviations

MR	Mineralocorticoid receptor
ER	Estrogen receptor
GR	Glucocorticoid receptor
AR	Androgen receptor
PR	Progesterone receptor
DBD	DNA binding domain
LBD	Ligand binding domain
SRC1	Steroid receptor coactivator1
PGC1α	PPARγ coactivator 1
PPARγ	Peroxisome proliferator-activated receptor gamma
NCOR2	Nuclear receptor corepressor 2
MRE	MR response element
HDAC	Histone deacteylase
DOCA	11-deoxycorticosterone
RAAS	renin-angiotensin-aldosterone-system
AT1R	Angiotensin II receptor type 1
ACE	Angiotensin converting enzyme
CCD	Cortical collecting duct

SGK	serum and glucocorticoid regulated kinase 1
ENaC	Epithelial sodium channel
CHF	Congestive heart failure
EF	Ejection fraction
MRA	Mineralocorticoid receptor antagonist
RALES	Randomized Aldactone Evaluation Study
EPHESUS	Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study
AMI	Acute myocardial infarction
EMPHASIS	Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure
EC-MR	Endothelial cell MR
EC	Endothelial cell
SMC	Smooth muscle cell
eNOS	endothelial nitric oxide synthase
NO	nitric oxide
cGMP	Cyclic guanosine monophosphate
Ach	acetylcholine
SKCa	small conductance calcium-activated potassium channel
IKCa	intermediate conductance calcium-activated potassium channel

ROS	reactive oxygen species
ET-1	endothelin-1
ET_A	endothelin A receptor
ET_B	endothelin B receptor
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
ICAM-1	intercellular adhesion molecule 1
VCAM-1	vascular adhesion molecule 1
ApoE	Apolipoprotein E
NF-κB	nuclear factor κB
TNF	tumor necrosis factor
MMTV	mouse mammary tumor virus
HEK	human embryonic kidney
ERE	estrogen response element
EC-MR-KO	endothelial cell-MR-knockout
$FENa^+$	fractional excretion of sodium
L-NAME	Nω-Nitro-L-arginine methyl ester hydrochloride
DBP	diastolic blood pressure
MAP	mean arterial pressure
SNP	sodium nitroprusside

LTCC	L-type calcium channel
TP	thromboxane prostanoid receptor

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Chapter 1. Introduction

The mineralocorticoid receptor

The mineralocorticoid receptor (MR) is member of the nuclear receptor family of hormone-activated transcription factors that is responsive to the steroid hormone aldosterone. Aldosterone activation of MR in the kidney regulates sodium and fluid balance thereby regulating blood pressure. The MR gene, NR3C2, has been mapped to chromosome 4 in humans and chromosome 8 in mice and spans 9 exons[1]. MR shares a common molecular structure with the family of steroid hormone receptors belonging to the nuclear receptor superfamily, including estrogen receptor (ER), glucocorticoid receptor (GR), androgen receptor (AR), and progesterone receptor (PR). MR possesses an N-terminal domain with ligand-independent activation function (encoded by exon 2), a DNA-binding domain (DBD) containing two zinc fingers that permit interaction with MR response elements in the promoter region of MR target genes (exons 3 and 4), a hinge region, and a ligand-binding domain (LBD) where aldosterone can bind and activate MR nuclear translocation and MR-mediated gene transcription (exons 5 through 9). While the DBD is conserved among steroid hormone receptors with especially strong homology between MR and GR, there is moderate variability among LBD to confer ligand specificity and very low homology in N-terminal domains.

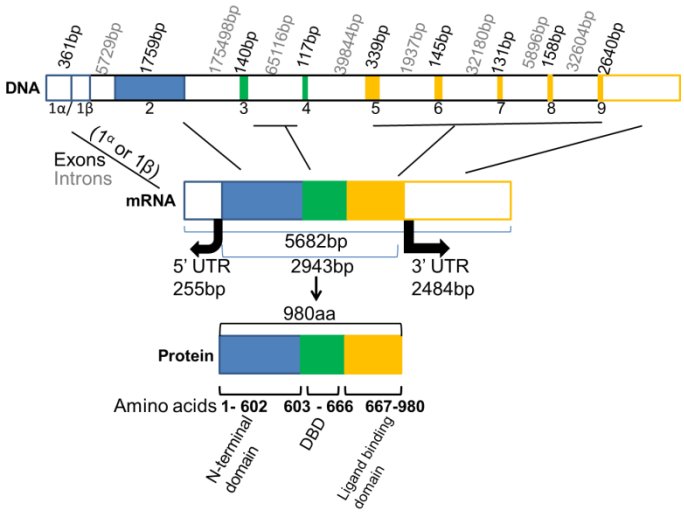

Figure 1.1. Mineralocorticoid receptor structure and functional domains.

In the absence of aldosterone, most of the MR is found in the cytosol in a complex with hsp90 and other co-chaperone proteins, which maintain MR in a conformation capable of binding ligand[2]. Aldosterone binding to MR causes exchange of co-chaperone FKBP51 for FKBP52, which binds the motor protein dynein, coordinating movement of this complex through the nuclear pore[3]. Once in the nucleus, MR can bind DNA and act as a transcription factor, activating or repressing expression of genes[4]. There is also evidence that nuclear receptors can be found in the nucleus in the absence of ligand[5] and that nuclear receptors are capable of regulating gene transcription in a ligand-independent manner[6]. Furthermore, a small proportion of MR is localized to the plasma membrane where it is activated by aldosterone and participates in non-transcriptional signal transduction by activating second messenger cascades and modulating phosphorylation events in the cell[7].

Unlike most steroid receptors, MR can be bound by multiple ligands. Cortisol, which circulates in the blood at concentrations 100 fold higher than aldosterone, readily binds the LBD of MR. In contrast to aldosterone, cortisol has been shown to be a partial agonist/antagonist of MR in multiple in vitro and in vivo studies[8-12]. However, the enzyme 11-ß-hydroxysteroid dehydrogenase type 2 (11-ß-HSD2) converts cortisol to its inactive metabolite, corticosterone, rendering aldosterone the primary ligand of MR in tissues expressing 11-ß-HSD2[1]. The importance of 11-ß-HSD2 was first characterized in renal epithelial cells but

has since been shown by multiple groups to be expressed in vascular smooth muscle cells and endothelial cells, demonstrating that aldosterone is an important physiologic ligand of MR in the vasculature[12-16].

Regulation of MR transcriptional activation

Regulation of MR activation and transcriptional activity is known to occur through several mechanisms. The N-terminal A/B domain of MR contains a ligand-independent transactivational function, AF-1 and is the least conserved among steroid receptors[17]. An early paper from Jausons-Loffreda et al that used a selection of A/B domain mutants to assess the role of the A/B domain in transcriptional activation of MR by aldosterone and cortisol in MCF7 cells found that while aldosterone-stimulated transcriptional activation was unaffected by loss of A/B domain regions, cortisol's ability to stimulate MR-dependent transcription was profoundly reduced in mutants specifically lacking residues 254-390[18], suggesting that the A/B region is specifically necessary for transactivation by cortisol..

The ligand-binding C-terminal domain of MR contains the transcriptional activation function-2 region (AF-2) and is an important location of coactivator binding and post-translational modifications that modulate MR function. Many nuclear receptor transcription factors, including ER, GR, and PR show upregulated transcriptional activation with binding of transcriptional coactivator steroid receptor coactivator 1 (SRC1)[19]. SRC1 binds nuclear receptors in a ligand-dependent fashion and increases histone acetylation via binding of additional cofactors such as p300/CBP[20]. A mammalian two-hybrid assay identified SRC1 subtype SRC1-4a as strongly binding the MR LBD in an aldosterone-dependent manner (1nM aldosterone) that was blocked by MR antagonist (MRA) eplerenone (10uM)[12]. The same study showed upregulated aldosterone-stimulated MR-mediated transcription of a luciferase reporter in the presence of SRC1 that was blocked with the addition of eplerenone. PGC1α, a well-described coactivator of nuclear receptors including PPARγ, was also identified as binding MR and enhancing transcriptional activation in this study, although to less strong effect. More recently the metallothionein tesmin has been identified as a transcriptional coactivator binding the AF-2 region of MR and enhancing aldosterone-stimulated transcriptional activation of MR in the setting of a luciferase reporter assay[21]. The transcriptional corepressor Nuclear Receptor Corepressor 2 (NCOR2, also referred to as SMRT) has conversely been shown to decrease MR-mediated transcription in response to aldosterone via histone deacetylaction[22].

Several post-translational modifications of MR capable of modulating MR activity have also been described. While histone acetylaction of MR response elements (MREs) enables increased MR binding and transcriptional activation, acetylation can also occur on MR itself, resulting in decreased transcriptional activation. Lee et al found that in vitro, pretreatment with a histone deacetylase inhibitor (HDACi) decreased MR and RNA polymerase II recruitment to MREs and decreased MR-mediated transcription of target genes, and that histone deacetylase 3 (HDAC3) enhanced MR-mediated transcription[23]. Furthermore, in mice made hypertensive by treatment with deoxycorticosterone acetate-salt treatment (DOCA, the mouse mineralocorticoid), treatment with an HDACi abolished hypertension and reduced enrichment of MR and Pol II on target gene promoters induced by DOCA[23]. Interestingly, while the histone acetyltransferase p300/CREB has been shown to be recruited to MR by SRC1, a study by Seo et al demonstrated that acetylation of MR itself by p300/CREB inhibits MR binding to target gene promoter regions and decreases MR-dependent transcription of renal epithelial genes in vitro[24]. These conflicting findings suggest the role of acetylation in modulating MR-mediated transcription depends on multiple factors, including available substrates of the acetyltransferases and deacetyltransferases.

Phosphorylation events also modulate MR-mediated transcription. An early work from Galigniana et al established that MR could be phosphorylated and that dephosphorylation increases DNA-binding capability of MR[25]. Shibata et al recently demonstrated that there is one phosphorylation site in the LBD of MR, S843, and that phosphorylation of this site by WNK4 renders MR unable to bind ligand[26]. Furthermore, physiologic stimuli associated with increased MR activation, such as low-salt diet, resulted in decreased phosphorylation of S843 and increased expression of mediators of electrolyte flux compared to normal chow, suggesting a role for phosphorylation of S843 in regulation of normal MR transcriptional function. Renin-angiotensin-aldosterone-system (RAAS) substrate angiotensin II is also capable of activating MR transcriptional activity as assessed by a luciferase reporter assay. In human coronary SMCs transfected with an MRE-luciferase reporter, angiotensin II increased MRE-luciferase activity in a dose-dependent manner[14]. This effect was revealed to require both the angiotensin type II receptor I (AT1R) and MR because it was blocked by the AT1R blocker losartan and the MR antagonist spironolactone.

Another factor in regulation of MR-mediated transcription is intramolecular binding within the MR. Interactions of the N- and C-terminal domains have been characterized in other nuclear receptors including ER[27], PR[28], and AR[29], with AR mutations in the regions required for this interaction correlating with androgen insensitivity in patients, suggesting a requirement for N/C interaction in AR transcriptional activation[30]. Rogerson and Fuller first showed that MR N-terminal and C-terminal peptides could interact using a mammilian-2-hybrid assay and then demonstrated the requirement of this interaction for aldosterone-stimulated transcriptional activation of full-length MR in COS-1 cells[31]. This stimulation was antagonized by spironolactone, eplerenone, and cortisol. A later paper from the same group confirmed that this interaction occurs through direct binding of the N- and C-terminal domains[32]. This finding reveals a structural requirement for MR transactivation and future studies must determine if this interaction has a role in the physiology and pathology of MR signaling in vivo.

There is some evidence in the literature for transcriptional regulation of steroid receptors by other steroid receptors. Based on the background that glucocorticoids have been shown to inhibit ERα+ breast cancer cell growth in vitro[33-35], Karmakar et al investigated the role of GR signaling in ERα-mediated MCF7 breast cancer cell proliferation. They found that GR-mediated repression of ERα-stimulated proliferaton of MCF7 breast cancer cells [36]. Specifically dexamethasone-stimulated GR inhibted ERα-mediated transcription of cell cycle genes pS2 and Cyclin D1 via direct protein-protein interaction between the C-terminal domain of GR and ERα. Hence there may be additional pathways regulating MR transcription that have not yet been elucidated.

Aldosterone-MR signaling in renal epithelial cells

The best characterized signaling pathway leading to MR activation is the RAAS pathway. In response to decreased renal perfusion, granular cells of the kidney's juxtaglomerular apparatus produce renin, an enzyme that circulates systemically and hydrolyzes liver-secreted angiotensinogen into angiotensin I[37]. Angiotensin I is further processed by angiotensin-converting enzyme (ACE) to angiotensin II. Angiotensin II counteracts low blood pressure by multiple pathways: it directly constricts blood vessels, leading to increased vascular resistance and increased blood pressure, and it also stimulates the zona glomerulosa cells of the adrenal gland to produce aldosterone[38]. Circulating aldosterone then binds renal epithelial

cytoplasmic MR in the cortical collecting duct (CCD) of the distal nephron of the kidney, triggering nuclear localization of the aldosterone-MR complex followed by receptor dimerization. MR can then bind MREs on promoter regions of target genes, recruit necessary cofactors and transcriptional machinery, and activate gene transcription[31]. The primary outcome of this pathway in the kidney is sodium reabsorption through apical epithelial channels coupled with potassium secretion, followed by passive movement of water, increasing blood volume.

The CCD of the distal nephron of the kidney is a site of regulation of electrolyte homeostasis via modulation of urinary electrolyte secretion. It has long been known that aldosterone regulates electrolyte transport in the tight epithelia of the CCD (reviewed in[39]). A 2000 paper from Náray-Fejes-Tóth demonstrated that aldosterone treatment increased mRNA of serine/threonine kinase serum-and glucocorticoid-regulated kinase (SGK) after 30 minutes in CCD cells[40]. This effect required MR as it was inhibited by an MR antagonist. This study also showed that SGK increased activity of epithelial sodium channel (ENaC), implicating SGK as an aldosterone-stimulated, MR-dependent modulator of Na^+ movement. Shortly following this paper, another group identified ENaC itself as a direct target of aldosterone-stimulated MR-mediated transcription[41]. These papers presented a molecular mechanism for the earlier research identifying aldosterone signaling through MR as mediating sodium and blood volume homeostasis.

Aldosterone-MR signaling in congestive heart failure

In addition to its physiologic role in regulating blood volume and consequently blood pressure, aldosterone-stimulated MR has long been known to be pathologically activated in many cardiovascular diseases, most dramatically in heart failure. There are two primary types of congestive heart failure (CHF): CHF with reduced ejection fraction (EF) due to an inability of the left ventricle of the heart to sufficiently eject blood into the peripheral circulation during systole, or CHF with preserved EF due to dysfunction of left ventricular filling[42]. Regardless of EF status, decreased cardiac output decreases arterial pressure, causing the blood volume sensing mechanism of the kidney's juxtaglomerular apparatus to activate the RAAS to increase blood volume. This results in inappropriately high levels of angiotensin II and aldosterone[43]. Increased angiotensin II levels cause vasoconstriction, leading to increased vascular resistance, and

aldosterone activates renal epithelial MR, resulting in sodium and water reabsorption and increased blood volume, further increasing resistance to the left ventricle's ejection of blood. This pathological RAAS activation exacerbates CHF and has identified RAAS signaling as a prime target in CHF therapy.

Multiple studies confirm the upregulation of RAAS substrates including aldosterone in the serum of CHF patients. A small study by Dzau et al demonstrated increased serum angiotensin II and aldosterone in patients with decompensated heart failure[44]. Swedberg et al then analyzed a larger study of patients with severe heart failure who had been enrolled in a randomized controlled trial of the ACE inhibitor enalapril in CHF and found that patients with baseline serum angiotensin II and aldosterone levels above the median had a nearly doubled risk of mortality after 6 months, compared to patients with levels below the median[45]. This study further observed that this increased risk was lost in the enalapril-treated group, demonstrating the effectiveness of inhibiting ACE in treating CHF. While ACE inhibitors transiently suppress angiotensin II-stimulated aldosterone production, ACE inhibition does not suppress long-term aldosterone production[46]. The phenomenon of "aldosterone escape", return of serum aldosterone levels to baseline or even increased serum levels during long-term exposure to ACE inhibitors, suggests there may be benefit in adding an MR antagonist to an ACE inhibitor in the setting of heart failure. The RALES trial sought to clarify this question.

MR antagonists in clinical trials

Much of the interest in investigating the role of MR in nonrenal tissues originates from clinical trials of MR antagonists (MRA) in cardiovascular disease. The RALES trial compared outcomes in patients with severe heart failure with decreased left ventricular ejection fraction receiving standard care (typically an ACE inhibitor, a loop diuretic, and digoxin) plus placebo with those receiving standard care plus spironolactone, an MR antagonist. Those receiving spironolactone had a 30% decreased risk of all-cause mortality and a 35% decreased risk of hospitalization over a 24-month period of follow-up[47]. These findings are especially striking in that they occurred "in the absence of a clinically significant hemodynamic effect" as described by the authors, and thus inhibition of natriuresis cannot account for the clinical benefit. Therefore, antagonism of nonrenal MR may contribute to the clinical benefit seen in RALES.

The MRA eplerenone, a more specific but less potent MR anatgonist, has been shown in the EPHESUS trial to decrease mortality when administered to patients following an acute myocardial infarction (AMI) in the setting of decreased left ventricular ejection fraction[48]. This trial showed a 15% decrease in mortality following AMI over a mean follow-up time of 16 months. In this trial, the natriuretic effect was statistically significant but clinically modest: over the course of the trial, mean systolic blood pressure increased by 8 mm Hg in the placebo group and by 5 mm Hg in the eplerenone group.

Most recently, the EMPHASIS trial compared placebo and standard care to eplerenone and standard care in patients with mild heart failure. This trial was also stopped early due to significantly decreased risks of mortality and hospitalization in patients in the treatment arm of the study[49]. Again, the effect of MRA on systolic blood pressure was statistically significant but modest: a mean decrease of 2.5 mm Hg in eplerenone-treated patients compared to a decrease of 0.3 mm Hg in the placebo-treated patients. Taken together, these trials provide strong support to the hypothesis that MR antagonism in non-renal tissues plays a role in the profound clinical benefit seen with MRA administration. Emerging evidence for the role of vascular endothelial cell MR (EC-MR) in cardiovascular disease supports the hypothesis that targeting of EC-MR by MRAs could contribute to the benefit seen in these clinical trials.

Vascular endothelium in cardiovascular health and disease

The vascular endothelium plays several crucial roles in maintenance of cardiovascular health and its dysregulation is known to contribute to cardiovascular disease. Vascular endothelial cells (ECs) form the innermost layer of the blood vessel, creating a monolayer barrier between the blood and the underlying smooth muscle cells (SMCs) and surrounding fibroblast- and extracellular matrix-rich adventitia. ECs perform a variety of vascular functions: providing a protective barrier to prevent platelet aggregation, participating in angiogenesis, modulating vasomotor function, and regulating inflammation in the vessel wall[50]. These last two will be discussed in the context of their significance to cardiovascular disease.

Role of vascular endothelial cells in vasomotor function

ECs play roles in both vasoconstriction and vasorelaxation, tightly regulated processes that contribute to regulation of regional blood flow and to blood pressure regulation by modulating vascular resistance. EC-

mediated vasodilation occurs in response to multiple stimuli. The enzyme endothelial nitric oxide synthase (eNOS) produces nitic oxide (NO), the best-characterized mediator of endothelial-dependent vasodilation. eNOS can be activated by several pathways. Flow-induced shear stress activates vascular endothelial growth factor receptor 2 in a ligand-independent manner, leading to activation of the phosphatidylinositol 3-kinase and consequently activation of the serine kinases Akt (protein kinase B) and protein kinase A (PKA). Akt and PKA phosphorylate eNOS on Ser1177, increasing its activity[51-53]. Alternatively, binding of acetylcholine (Ach) or bradykinin to their EC receptors results in activation of phospholipase C, leading to production of inositol triphosphate and subsequent release of Ca^{2+} from the endoplasmic reticulum into the cytosol[54]. Increased intracellular Ca^{2+} has multiple pro-vasodilatory consequences in endothelial cells[54]. First and best characterized is the binding of Ca^{2+} to calmodulin, required for activation of eNOS. eNOS catalyzes the conversion of L-arginine to L-citrulline and NO. NO diffuses from ECs to the SMCs where it activates soluble guanylate cyclase, which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). cGMP acts as a second messenger, binding to regulatory subunits of protein kinase G to activate protein kinase G, which then phosphorylates myosin light chain phosphatase to inhibit its action. Myosin light chain phosphatase dephosphorylates myosin, a necessary step in its cyclic interaction with actin to allow SMC contraction[55].

A second pathway by which EC-dependent vasodilation can occur is through production of prostacyclin. In response to increased intraceullular Ca^{2+}, arachidonic acid is released and metabolized by cyclooxygenase to various products including prostacyclin[56]. Prostacyclin is released from ECs and binds its receptor on SMCs, leading to increased cAMP, activation of protein kinase A, and increased phosphorylation of myosin light chain phosphatase, resulting in increased myosin light chain phosphatase activation and vasorelaxation[57].

The third EC-dependent pathway regulating vasodilation takes advantage of the potential for electrical coupling between the EC layer and the SMC layer via electrically conductive processes called myoendothelial gap junctions[58;59]. When intracellular Ca^{2+} is increased in response to Ach or bradykinin, Ca^{2+}-activated potassium channels small conductance potassium channel (SKCa) and intermediate conductance potassium channel (IKCa) in the ECs. These channels open to allow efflux of K^+ out of the

cell, hyperpolarizing the endothelium and allowing hyperpolarization to propagate along the myoendothelial gap junctions, decreasing the ability of the SMC to contract. NO has also been shown to directly activate large-conductance calcium-activate potassium channels on SMCs, causing hyperpolarization of the vessel wall and decreasing its ability to contract[60].

ECs also participate in regulation of vasoconstriction via vasoconstrictor production. Endothelin-1 (ET-1) is an potent vasoconstrictive peptide produced by ECs in response to inflammatory stimuli including hyperlipidemia, hypoxia, and reactive oxygen species (ROS)[61]. When ET-1 binds its receptors endothelin A receptor (ET_A) and endothelin B receptor (ET_B) on vascular SMCs, it triggers a strong, sustained vasoconstrictive response. In contrast, ET-1 binding to endothelial ET_B elicits vasorelaxation via increased NO production and endothelin-1 degradation[62].

To examine the specific role of EC-MR in blood pressure regulation and vasoreactivity, recent studies have utilized transgenic animals in which MR expression has been modulated in the endothelium (Table 1.1). Mice overexpressing human MR specifically in ECs have elevated blood pressure, increased mesenteric myogenic tone and constriction, and no change in mesenteric endothelial-dependent vasodilation[63]. However, two groups recently deleted MR from ECs using a Tie2 promoter strategy revealing no difference in basal blood pressure or vasoconstriction[64;65]. Using this model, Rickard et. al. demonstrated decreased aortic and mesenteric endothelial-dependent relaxation[64], while Schäfer et. al. found no change in aortic endothelial function in healthy animals but saw protection from obesity-associated aortic endothelial dysfunction in the EC-MR knockout (EC-MR-KO)[65]. In addition to these conflicting results, these EC-MR-KO mouse models are confounded by MR deletion from leukocytes by the Tie2 promoter[65], making interpretation difficult due to the recent identification of a role of leukocytes in blood pressure regulation[66] and a role for MR in regulating leukocyte function[67].

Role of endothelial function in cardiovascular disease

Dysregulation of the processes contributing to endothelial-dependent vasodilation is broadly termed "endothelial dysfunction". Endothelial dysfunction has long been associated with vascular disease, with many studies showing impaired endothelial-dependent relaxation in patients with hypertension[68-70].

Whether endothelial dysfunction is a cause of hypertension remains controversial. Some studies support that endothelial dysfunction predisposes people to later development of hypertension, including a study of postmenopausal women demonstrating that baseline endothelial dysfunction predicted the future risk of developing hypertension[71]. Also, NO levels have been shown to be decreased in individuals with hypertension[72;73] and NO-synthesis inhibitors lead to hypertension in animal models[74;75]. Other studies refute this link. In a large study of normotensive individuals, baseline brachial artery flow-mediated dilation (a measure of endothelial function in humans) did not predict subsequent development of hypertension[76]. Another group found evidence of the opposite, that increased blood pressure actually predicts later development of endothelial dysfunction in a smaller cohort of adolescent boys [77]. Regardless of whether endothelial dysfunction causes hypertension or occurs secondarily to it, it is an independent risk factor for survival in cardiovascular disease[78;79]. Furthermore, there is evidence for involvement of MR in endothelial function in the setting of disease. MR antagonists have been shown to restore endothelial-dependent relaxation in animal models of vascular disease including streptozocin-induced diabetic rats[80] and Wistar rats exposed to the coronary artery ligation model of MI[81]. Likewise, MRAs ameliorate endothelial dysfunction in patients in the setting of hypertension[82], heart failure[83], type II diabetes[84], and chronic kidney disease[85]. Thus mineralocorticoid receptor antagonist targeting of endothelial MR may provide substantial clinical benefit.

Oxidative stress has been shown to play a significant role in development of endothelial dysfunction. Production of ROS including superoxide, the hydroxyl radical, and peroxynitrite takes places in endothelium catalyzed by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase, enzymes of the mitochondrial electron transport chain, and other enzymes. ROS production occurs at very low levels in healthy tissue but is upregulated in vascular disease[86]. Superoxide reacts with available NO to form peroxynitrite, decreasing NO available for maintenance of vasodilation. Many studies support a role for MR in production of ROS. In vitro endothelial cell studies indicate a principally pro-ROS, anti-NO effect of MR activation by aldosterone following hormone treatment between 3 and 24 hours[87;88], while one study assessing the effect of aldosterone after ten minute treatment found increased NO production[89]. In a rat model of CHF with aortic endothelial dysfunction, spironolactone treatment decreased superoxide production, increased eNOS expression in the aorta, and helped to restore endothelial-dependent

relaxation[90]. Two studies using mineralocorticoid-salt models of rat hypertension showed similar upregulations in ROS production that could be blocked by eplerenone[91] or the NADPH oxidase inhibitor apocynin[92]. One study of macrophages isolated from patients with CHF compared to healthy controls showed that while aldosterone increased ROS production and addition of eplerenone attenuated this increase in both groups, eplerenone alone did not affect ROS in macrophages from healthy people while it decreased ROS in cells from CHF patients[93]. In a study of chronic kidney disease patients, those receiving eight weeks of spironolactone treatment had significantly reduced urinary isoprostanes, a marker of ROS activity, compared to those receiving placebo[94]. Therefore in vitro studies, animal studies, and human data support that MR modulates production of ROS and that this contributes to impaired endothelial eNOS function. Less is known about the impact of ROS on other mediators of endothelial-dependent vasodilation, but ROS has also been shown to reduce prostacyclin production by inducing tyrosine nitration of prostacyclin synthase[95]. Likewise, activity of the Ca^{2+}-activated K^+ channels SKCa and IKCa is attenuated by superoxide. ROS also reduce current between ECs and SMCs via myoendothelial gap junctions[96]. Thus MR-stimulated ROS production may attenuate these activators of endothelial-dependent vasodilation as well.

The endothelial-derived vasoconstrictor ET-1also plays a role in cardiovascular disease, particularly in regulation of the coronary circulation. Plasma ET-1 levels inversely correlate with coronary flow reserve 24 hours after AMI[97]. Plasma ET-1 also predicts impaired coronary reflow after percutaneous coronary intervention following AMI [98]. Furthermore, coronary microvessels distal to stenosis show enhanced vasoconstrictive response to ET-1[99]. In CHF patients, elevated concentrations of ET-1 predict mortality and hospitalizations[100]. Some evidence suggests a link between MR activation and ET-1 regulation. Aldosterone stimulates transcription of the gene for ET-1, *edn1*, in rat renal collecting duct cells in an MR-dependent manner[101]. It has also been shown that aldosterone decreases endothelial ET_B-stimulated NO production in a mouse model of pulmonary hypertension via oxidation of a cysteine in the eNOS-activating region of ET_B, and this effect was inhibited by spironolactone[102]. If MR activation enhances ET-1 signaling and inhibits endothelial ET_B-mediated opposition of vasoconstriction, this may offer a potential mechanism for the protection seen with MRAs following AMI.

Role of endothelial cells in atherosclerosis

ECs and EC-MR also play a significant role in vascular inflammation and atherosclerosis. Atherosclerosis is a chronic progressive inflammatory disease of the vasculature in which increased serum lipoproteins accumulate within the wall of the blood vessel. As atherosclerosis develops, endothelial cells undergo multiple pathological processes. In its early stages, hyperlipidemia can lead to endothelial dysfunction via several mechanisms including superoxide-mediated modification of NO[103], increased production of asymetric dimethylarginine[104;105], an endogenous competitive inhibitor of NO synthase, and oxidation of low density lipoprotein[106;107]. As lipoproteins become oxidized, components of the lipoproteins can also activate expression of EC adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1), enabling infiltration of immune cells into the plaque[108-110]. As leukocytes accumulate within the plaque, releasing additional pro-inflammatory chemokines that attract more leukocytes, a fibrous cap of smooth muscle cells forms between the ECs and the plaque itself. As the lipid and inflammatory content of the plaque increases, protease activity renders the plaque increasingly unstable and susceptible to rupture, leading to plaque surface thrombosis and obstruction of blood flow[111]. This thrombosis can cause myocardial infarction and stroke, two leading causes of death.

Role of MR in atherosclerosis

MR activation substantially contributes to atherosclerosis development and progression. Epidemiologic studies indicate that serum aldosterone levels are an independent risk factor for cardiovascular ischemia[112 113]. A study of patients with coronary artery disease demonstrated an association between increased serum aldosterone levels, even within the normal range, and increased risk of MI or cardiac death[114]. Animal models support a causative relationship between MR activation and exacerbated atherosclerosis. In these studies, aldosterone infusion enhanced atherosclerotic plaque burden, while MR antagonism reduced plaque size and inflammatory marker expression[115-118]. In the atherogenic Apoliporotein E (ApoE) knockout mouse model of atherosclerosis, MR activation via sub-pressor dose aldosterone infusion enhanced leukocyte infiltration into athero-prone regions of the vasculature, exacerbating plaque development [119]. MR activation in multiple cell types could enhance atherosclerosis. However, as the endothelium mediates the ability of immune cells to migrate into the blood vessel, one potential hypothesis

to account for the enhanced leukocyte infiltration seen with aldosterone infusion would be that MR activation in the ECs enhances leukocyte infiltration. It has already been shown that aldosterone activates transcription of pro-inflammatory genes in ECs in an MR-dependent manner including the adhesion molecule ICAM-1[120]. Furthermore, aldosterone-mediated ICAM-1 induction contributes to enhanced leukocyte adhesion to cultured human coronary ECs. Thus, by enhancing leukocyte-endothelial cell adhesion, EC-MR may contribute the exacerbation of vascular inflammation and atherosclerosis induced by aldosterone in vivo.

Role of ERα in atherosclerosis

While MR activation exacerbates cardiovascular pathology, activation of ERα, an estrogen-activated steroid hormone receptor, generally ameliorates it. Estrogen treatment decreased serum lipids and atherosclerotic lesion size in the ApoE-/- mouse model of atherogenisis[121-123]. Similarly in humans, in one study of patients under 45 years of age, female sex was associated with significantly decreased risk of coronary artery disease[124] assessed angiographically. ERα activation also regulates endothelial function and mediates the role of ECs in atherosclerosis. Ovariectomized animal models develop endothelial dysfunction that is reversed with exogenous estrogen treatment through enhancement of eNOS activity [125-127]. In postmenopausal women, chronic estrogen treatment increases vasodilation[128]. These findings correlate with epidemiologic studies consistently showing a relative protection from heart attack and cardiovascular mortality in premenopausal women compared to men of the same age, a protection that is lost after menopause[129]. In vitro, estrogen downregulates endothelial expression of adhesion molecule VCAM-1via inhibition of transcription factor NF-κB[130] and inhibits leukocyte adhesion to TNF-α-activated endothelial cells by attenuating production of pro-inflammatory cytokines[131]. In vivo, estrogen treatment decreased leukocyte adhesion and transendothelial migration in hyperlipidemic rabbits[132] and attenuated VCAM-1 expression and plaque size in mice[133]. The specific role of intact endothelium in mediating ERα's anti-atherogenic effect was confirmed in a study in which hyperlipidemic rabbits exposed to endothelial denuding by wire injury were no longer protected from atherosclerosis by estrogen treatment[134]. An elegant experiment from Billon-Gales et al demonstrated that EC-ERα is necessary for the protective effect of estrogen in atherosclerosis. This group used an endothelial-specific knockout of ERα using the Tie2-Cre

promoter crossed with the LDLr-/- atherogenic mouse to assess the role of EC-ERα in vivo, and found that mice lacking EC-ERα were no longer protected from plaque development by estrogen administration[135].

Model	Reference	Blood pressure	Vasoconstriction	Vasorelaxation	Method
EC-specific hMR overexpression	Nguyen Dinh Cat, FASEB, 2010	Elevated at baseline; elevated in response to Angiotensin II & ET-1	Enhanced in mesentery to KCl, PE*, Tbx agonist, Angiotensin II, ET-1; intraluminal pressure (MT)	ND in mesentery to Ach or SNP	Wire & pressure myography
Tie2-Cre EC MR KO	Schäfer, European Heart Journal, 2013	Not assessed	ND in aorta to NE or KCl*	ND in healthy aorta to Ach or SNP; on 14 wk HFD, MR-intact showed ↓Ach response but KO was protected	Wire myography
Tie2-Cre EC MR KO	Rickard, Hypertension 2014	No difference at baseline or on 8 weeks DOCA/salt/uninephrectomy	ND in aorta or mesentery to Tbx agonist U46619*	↓mesenteric and aortic relaxation to Ach in KO; ND to SNP	In aorta: wire myo In mesentery: pressure myo

Table 1.1. Transgenic studies exploring the role of EC-MR in blood pressure regulation and vasomotor function.

Chapter 2. Endothelial mineralocorticoid receptor function is inhibited by estrogen receptor

Introduction

Epidemiologic studies have consistently demonstrated a relative protection from heart attack and cardiovascular mortality in premenopausal women compared to men of the same age. This protection is lost after the age of menopause[129], yet the mechanisms for it remain poorly understood. Much evidence supports a role for endogenous estrogen in mediating this protective effect[136;137]. In contrast to the many beneficial cardiovascular effects seen with activation of ERα by E2, activation of MR by aldosterone exacerbates cardiovascular disease[119;138-140]. In humans, elevated serum aldosterone levels correlate with increased incidence of heart attack, stroke, and cardiovascular death in patients with hypertension or other cardiovascular risk factors[114;141]. Conversely, MR antagonists prevent cardiovascular mortality[47-49;142] by unclear mechanisms. In animal models of hyperlipidemia-induced atherosclerosis or wire-induced vascular endothelial damage, aldosterone infusion promotes atherosclerosis and vascular remodeling after injury[119;143] while estrogen is protective in the same animal models[144-146].

Endothelial dysfunction represents an early stage of vessel wall damage and is a marker of subclinical atherosclerosis[147;148] . Human studies demonstrate that endothelium-dependent vasodilation and subsequent progression to atherosclerosis correlate with plasma aldosterone levels, suggesting that aldosterone action specifically in the endothelium may be important in the pathogenesis of atherosclerosis[149]. Likewise, multiple human studies reveal an association of serum estrogen levels with preservation of endothelial function in woman[150-152]. Even in men, estrogen level is positively associated with flow-mediated vasodilation independent of other cardiovascular risk factors[153]. Based on these data, we hypothesized that estrogen signaling through ERα may protect the vasculature by inhibiting the detrimental effects of aldosterone and MR on the regulation of genes in endothelial cells that contribute to cardiovascular disease. To test this hypothesis we investigated the interaction between ER and MR transcriptional function using a heterologous promoter system in vitro, examined interactions between the receptors by biochemical assays,

determined the impact of ER on MR regulation of ICAM-1 expression in vascular endothelial cells, and assessed the relevance of our findings using an in vitro functional assay of immune cell adhesion to the endothelium.

Results

The Estrogen Receptor Inhibits Mineralocorticoid Receptor-Mediated Transcriptional Activation

To test whether an interaction exists between the transcriptional activity of ER and MR, transcriptional reporter assays were performed in HEK293 cells using a luciferase reporter containing the mouse mammary tumor virus (MMTV) MR-responsive element (MRE-Luciferase, Figure 2.1A) or containing the Xenopus vitellogenin ERE24, an ER-responsive element (ERE Luciferase, Figure 2.1B). Since these HEK293 cells do not express endogenous MR (or GR), aldosterone (and cortisol) did not affect MRE-luciferase reporter activity in the absence of cotransfected MR (Figure 2.2). Expression of MR increased MRE-Luciferase reporter activity likely due to ligand-independent MR activation. MRE activity was further augmented by
addition of aldosterone in a dose-dependent manner. We began with 10 nM, as this is concentration consistent with that found in the serum of patients with cardiovascular disease[154]. Cotransfection of an ERα-expressing plasmid substantially attenuated MR-dependent reporter activity and further addition of estrogen completely inhibited MRE reporter activity (Figure 2.1A).

ERE-Luciferase reporter activity was unaffected by co-transfection with MR alone or with aldosterone at 10nM (data not shown) or 100nM (Figure 2.1B) even with higher levels of MR expression (data not shown) as confirmed by immunoblotting (Figure 2.3). These data demonstrate that the ER, particularly in the presence of estrogen, attenuates MR transcriptional activity but that the converse is not true as MR does not influence ER genomic activity under the same conditions.

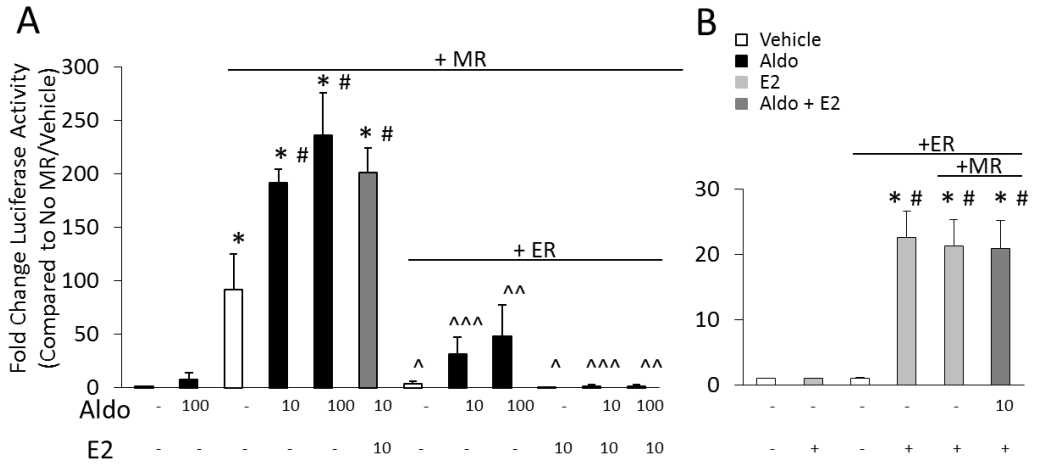

Figure 2.1. Estrogen inhibits MR-mediated transcriptional activity. (A) Estrogen receptor (ER) inhibits mineralocorticoid receptor (MR)-mediated transcriptional activation by aldosterone. HEK293 cells were transfected with the MR-responsive MMTV-luciferase reporter. Aldosterone stimulation (10 nM or 100 nM, as indicated) of MR transcriptional activity is significantly attenuated by expression of the ER and completely inhibited by estrogen (E2, 10nM) with ER. (B) No effect of MR on ER-stimulated transcription. HEK293 cells were transfected with a luciferase reporter driven by an ER-responsive element. E2 (10nM) induction of ER-mediated reporter activity was unaffected by co-transfection with MR with or without aldosterone (100nM). N=3 experiments. *$p<0.001$ versus no MR (A) or no ER (B), #$p<0.01$ versus MR or ER + vehicle. ^$p<0.05$ versus MR + vehicle, ^^$p<0.01$ versus MR + 100 nM aldosterone, ^^^$p<0.001$ versus MR + 10 nM aldosterone.

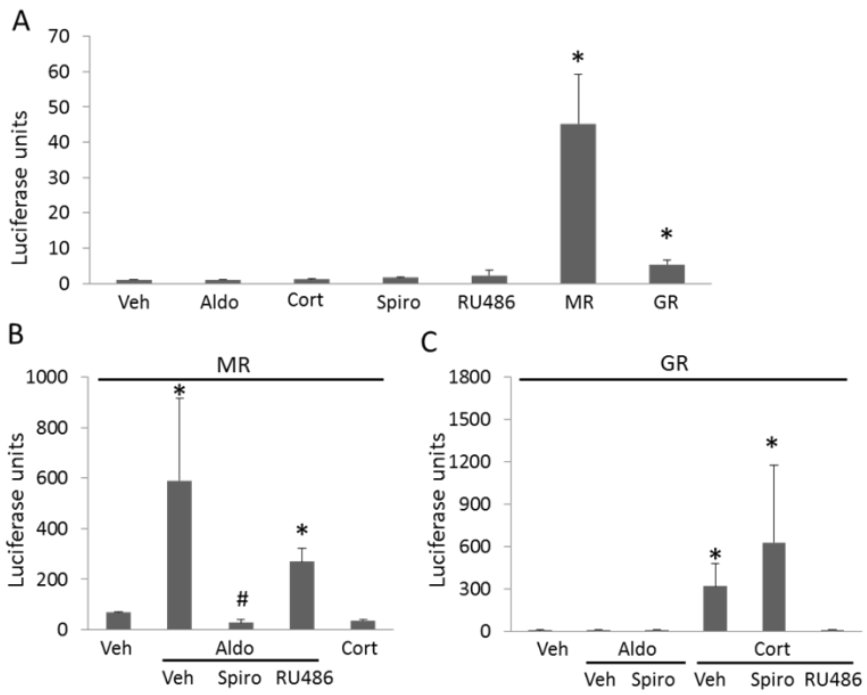

Figure 2.2. Characterization of the MMTV-luciferase reporter in HEK293 cells. (A) HEK293 cells were transfected with a reporter plasmid containing the mouse mammary tumor virus (MMTV) long terminal repeat driving expression of the luciferase gene and treated with vehicle (Veh), the indicated hormone or inhibitor, or transfected with plasmid expressing the mineralocorticoid receptor (MR) or the glucocorticoid receptor (GR). Luciferase response was quantified and expressed as fold change over vehicle-treated cells transfected with empty expression vector. Lack of luciferase response to 10 nM aldosterone, 10 nM cortisol (Cort), 1 micromolar (uM) spironolactone (Spiro, MR antagonist), or 1 uM RU486 (GR antagonist), reflects the lack of endogenous MR and GR expression in these HEK293 cells. (B) MR expressed in HEK293 cells can be transcriptionally activated by either aldosterone or Cort treatment. (C) In HEK293 cells expressing only transfected GR, reporter activity is enhanced by Cort and unaffected by 10 nM aldosterone. * $p < 0.05$ versus vehicle; # $p < 0.05$ versus aldosterone, N = 5 experiments.

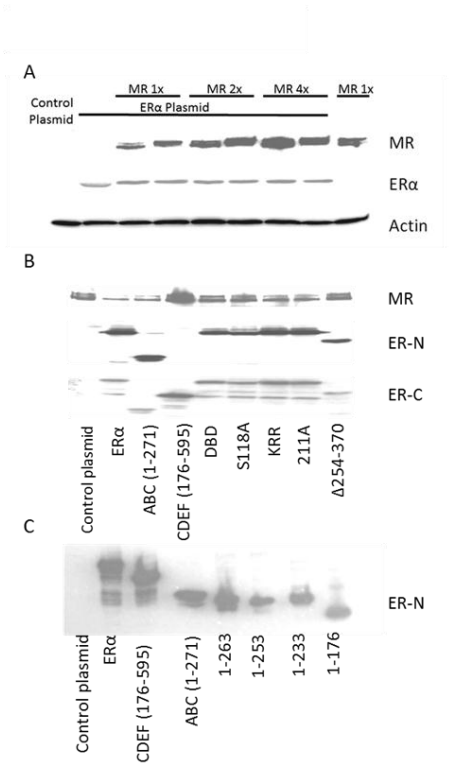

Figure 2.3. Confirmation of receptor expression in HEK293 cell reporter assays. (A) HEK293 cells were transfected with a luciferase reporter driven by an ER-responsive element (ERE) alone or along with estrogen receptor α (ERα) and increasing concentrations of MR expression plasmid (1x, 2x, or 4x the amount of ER plasmid). The level of ER and MR expression in lysates from the experiment shown in Figure 1B are shown by immunoblot (IB). ER and 1x MR levels are used throughout Figures 1 and 2. Higher levels of MR expression (2 and 4 fold greater than ER plasmid) were also tested and there was no effect of MR and aldosterone on ERE reporter activity despite substantial MR expression as demonstrated here. (B-C) Confirmation of expression of ER mutants in Figure 2. HEK293 cells were transfected with a luciferase reporter driven by an MR-responsive element (MRE) alone or along with mineralocorticoid receptor (MR) and various estrogen receptor α (ERα) mutant constructs. (B) Expression of MR and ER mutants is confirmed in lysates from experiments shown in Figure 2B. MR antibody demonstrates expression in all cases with some variability in quantity of MR that does not correlate with ER inhibition of reporter activity. Using the ERα N-terminal antibody (ER-N) the correct size and expression of all mutants is confirmed with the exception of CDEF (176-595) that lacks the N-terminal antigen. Expression of CDEF along with other mutants is confirmed using an ER C-terminal antibody (ER-C). (C) Expression of the

ER C-terminal deletion mutants is confirmed in lysates from experiments shown in Figure 2C using the ER-N antibody.

MR transcriptional inhibition by ER is mediated by the ER N-terminus and does not require ER DNA binding or non-genomic signaling

To determine the ERα functional domains that are necessary for inhibition of MR-induced transcription, MRE-Luciferase reporter assays were performed in the presence of ERα with mutated functional domains (Figure 2.4A, top). ERα with mutations that delete the C-terminus (ABC) or the N-terminus (CDEF) or that specifically inactivate DNA binding (DBDmut), ligand independent activation (S118A), or rapid non-genomic signaling (KRR), all maintain the ability to inhibit MR-mediated transcription. However, a mutant lacking amino acids 254 to 370, including the ERα hinge region and the nuclear localization signal, is insufficient to inhibit MR transcriptional activity (Figure 2.4B) despite relatively equal expression of all ER mutants (Figure 2.3B). This domain alone (253-370) is also insufficient to significantly inhibit MR transcriptional activity (Figure 2.4C) suggesting that additional domains are also needed.

As the ERα N-terminal ABC domain (amino acids 1-271) was sufficient to inhibit MRE-luciferase activity, additional deletion mutants of the ERα N-terminus were generated to identify the portion of ERα that is necessary to inhibit MR-mediated transcription (Figure 2.4A, bottom). MRE luciferase reporter assays in Figure 2.4C reveal that ERα amino acids 1-253 is sufficient to mediate MR transcriptional inhibition while deletion of 20 additional ERα C-terminal amino acids from 253 to 233 completely prevented ERα inhibition of MR function despite similar expression of each ERα deletion mutant (Figure 2.3C).

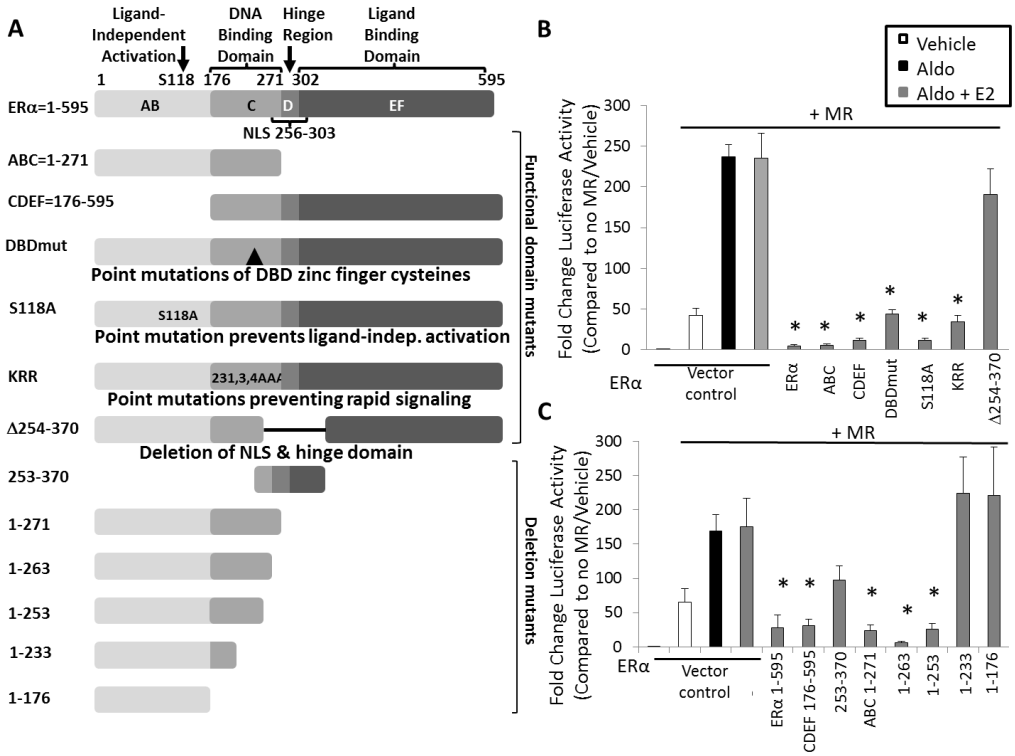

Figure 2.4. ERα functional domains that contribute to MR transcriptional inhibition. (A) Schematic of full length ERα alongside functional and deletion mutants. The depicted ERα constructs were co-transfected with MR into HEK293 cells and MRE-luciferase reporter activity was quantified. (B) ERα mutants inhibited MR transcriptional activity despite lacking the AB domain (CDEF) or the ligand binding domain (ABC) or with point mutations that inactivate DNA binding (DBDmut), ligand-independent activation (S118A), or non-genomic signaling (KRR). A deletion mutant lacking the nuclear localization signal (NLS) and hinge region (Δ254-370) did not inhibit MR transcription; however, this domain alone (253-370) was insufficient to significantly inhibit MR transcriptional activity (see C). (C) ERα amino acids 1-253 without the ligand binding domain was sufficient to inhibit MR transcriptional activity. DNA binding domain=DBD. N=5 experiments. *$p<0.05$ versus MR+Aldosterone+E2.

MR and ERα are part of a protein complex in cells

To further explore the mechanism by which ER inhibits MR activity, we examined whether ER and MR are part of a common protein complex in cells. MR was immunoprecipitated from cell lysates from HEK293 cells transfected with full length human MR and ERα, and the presence of ERα in the complex was determined by immunoblotting for ERα. Indeed, abundant ERα protein is present in the MR immunoprecipitated complex (Figure 2.5A). When MR-expressing HEK293 cell lysate was incubated with His-tagged ERα, the ERα-containing His beads pull down a complex that contains MR (Figure 2.5B). While this experiment does not test for a direct interaction between the two receptors, the data support the conclusion that MR and ERα are simultaneously part of a protein complex in cells. In addition to their role in transcriptional regulation in the nucleus, both ER and MR have been shown to function in a rapid, "nongenomic" manner in the cytoplasm tethered to the membrane by the scaffolding protein striatin[155;156]. To test whether the ER-MR complex is mediated by interaction of the two receptors with striatin, the His-ERα pull down was repeated using a His-tagged ERα with mutations in three amino acids critical for striatin binding and rapid signaling (KRR mutant[157]). ERα with the KRR mutation does not pull down striatin as expected, but is still able to pull down a complex containing the MR (Figure 2.5C) suggesting that striatin is not necessary for ER-MR complex formation . The role of the hormonal ligands in the ER-MR complex formation was also explored using lysates from HEK293 cells expressing ERα and MR and treated with the indicated hormones. ER and MR are present in a common complex in the absence and presence of either aldosterone or E2 and the ratio of the receptors in the complex is not altered by treatment with aldosterone. Treatment with E2 alone significantly increased the amount of ER in the complex with the MR although this difference is not present in cells treated with E2 together with aldosterone (Figure 2.5D).

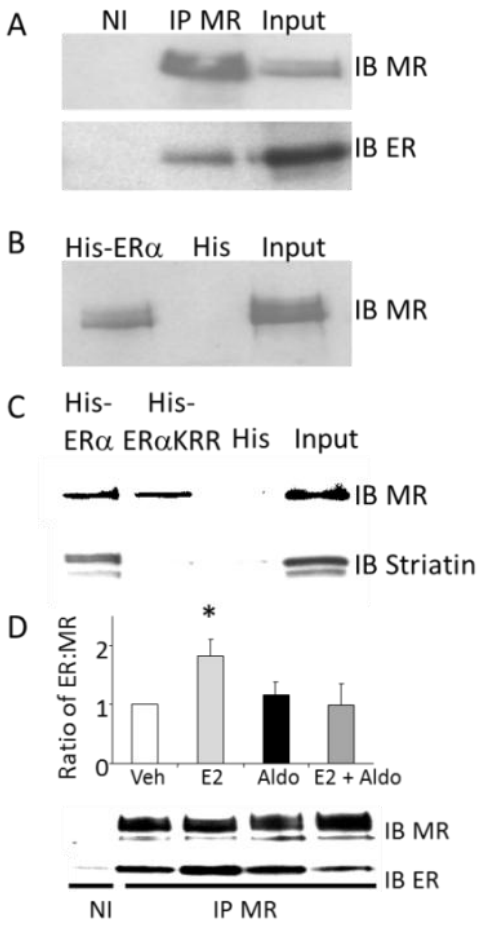

Figure 2.5. ER and MR are part of a protein complex in cells. (A) Cell lysates from HEK293 cells expressing full length human MR and ERα were immunoprecipitated (IP) with anti-MR antibody or control non-immune (NI) serum and the complex was immunoblotted (IB) with anti-MR or anti-ERα antibody. (B) His-ERα beads or His beads alone were incubated with MR-expressing HEK293 cell lysate and the bound complex was immunoblotted (IB) to detect the presence of MR. (C) Striatin binding is not required for ER to form a complex with MR. Beads with wild type ERα, His-ERα-KRR (the mutant that cannot bind to striatin thereby preventing rapid non-genomic ER signaling), or His beads alone were incubated with MR-containing HEK293 cell lysates followed by immunoblotting for MR or striatin. (D) E2 treatment increases ERα complexing with MR. The role of the ligand in the ER-MR complex formation was examined by treating HEK293 cells expressing MR and ER with 10nM E2 and/or 100nM aldosterone followed by IP of MR from the lysate and IB for MR or ER. N=4 experiments. *$p<0.01$ versus vehicle.

Both complex formation with MR and nuclear localization may be necessary for ERα inhibition of MR-mediated transcription

We next examined the domains required for ER-MR complex formation. His-tagged ERα domains corresponding to the mutants that determined MR transcriptional inhibition in Figure 2.4 were incubated with lysate from HEK293 cells expressing MR followed by immunoblotting of the bound proteins for MR (Figure 2.6A). As in the reporter assays, ERα lacking the AB domain (amino acids 176-595) or with the ligand-binding domain deleted (amino acids 1-271) were each able to form a complex containing the MR. Interestingly, deletion of the hinge region containing the NLS (delta 254-370) did not prevent ER interaction with MR in a whole cell lysate despite the finding that this deletion mutant did not inhibit MR transcriptional activity in cells in vitro. However, this ERα domain alone (254-370) was insufficient to complex with the MR binding (Figure 2.6A). Further deletions of the ERα N-terminus demonstrated that amino acids 1-253 and 1-233 are sufficient for MR complex formation, but that further deletion to 1-176 prevents complex formation with the MR. Comparison to the reporter assay reveals some similarities but also some differences in the domains involved in the ER-MR complex formation (Figure 2.6C, "ER-MR complex") and transcriptional inhibition (Figure 2.6C, "Inhibits MRE Reporter"). Since MR transcriptional activity occurs in the nucleus, we next investigated which of the ERα mutants can localize to the nucleus in the presence of E2. HEK293 cells transfected with flagged-tagged ERα deletion mutants were treated with E2 and the localization of ERα determined by immunfluorescent staining with antibody to the ERα epitope tag and DAPI to mark the nuclei. As expected, in the presence of E2, full-length ERα (1-595) localizes to the nucleus, while cells expressing ERα with the NLS deleted (Δ254-370) showed diffuse cytoplasmic ERα signal that does not co-localize with the DAPI-stained nucleus (Figure 2.6B). ERα 1-271 is nuclear while cells expressing ERα 1-253 showed both nuclear and cytoplasmic localization of the receptor and cells expressing ERα 1-233 had predominantly cytoplasmic staining (Fig 2.6B). The observations that ERα 1-233 binds to MR in lysed cells but lacks strong nuclear localization and does not inhibit MR-mediated transcription supports the possibility that ERα inhibition of MR-mediate transcription requires the concentration of ERα in the nucleus where it can subsequently form a complex with the MR. Indeed, further exploration of the subcellular localization of ERα mutants (Figure 2.7) combined with the MR

binding and reporter activity data reveals that the ability of ERα to inhibit MR-mediated gene transcription correlates with the ability of ERα segments to both localize to the nucleus and to form a complex with MR (Figure 2.6C).

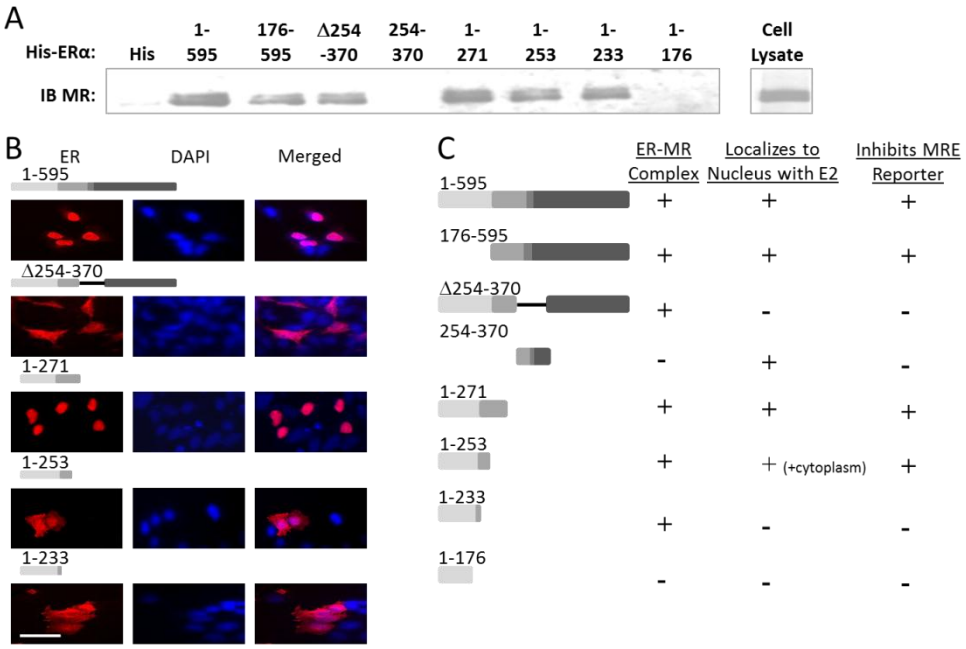

Figure 2.6. ERα domains that bind MR and also localize to the nucleus with E2 treatment are sufficient to inhibit MR transcriptional activity. (A) His beads containing ERα deletion mutants of varying lengths were mixed with lysate from HEK293 cells expressing MR and the bound proteins were immunoblotted (IB) with anti-MR antibody. (B) HEK293 cells transfected with flag-tagged ERα mutants were treated with 10nM E2 followed by immunoflourescent microscopy with anti-flag antibody (red) and DAPI to indicate the nuclei (blue). Merged images show nuclear versus cytoplasmic localization. Scale bars, 25μM. (C) Summary of ERα domains capable of complexing with MR (from Figure 4A), localizing to the nucleus with E2 (from Figure 2.6B and Figure 2.7), and inhibiting MR response element (MRE) reporter activity (from Figure 2). Only mutants that form a complex with MR and also localize in the nucleus where MR genomic activity takes place, are able to inhibit MRE-luciferase reporter activity.

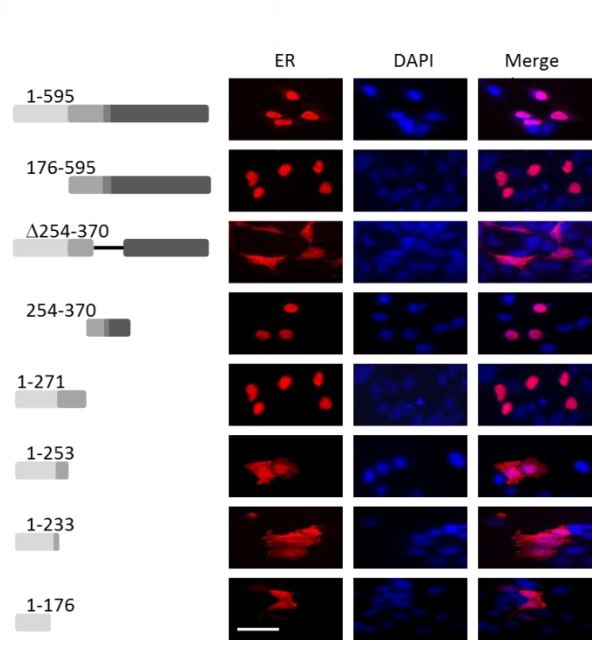

Figure 2.7. Cellular Localization of ERα deletion mutants. HEK293 cells transfected with flag-tagged ERα mutants of varying length were treated with 10nM E2 followed by immunoflourescent microscopy with anti-flag antibody (red) and DAPI to indicate the nuclei (blue). Merged images show nuclear versus cytoplasmic localization. Full length ERα localizes to the nucleus as does C-terminus segment 176-595, the N-terminus segment 1-271, and the fragments 254-370 and 176-271. ERα Δ254-370 lacking the nuclear localization sequence fails to show nuclear localization. ERα 1-253 shows nuclear and cytoplasmic localization and ERα 1-233 and 1-176 are predominantly cytoplasmic.

In human endothelial cells, estrogen inhibits aldosterone induction of MR transcriptional activity and of intercellular adhesion molecule-1 (ICAM-1) expression

Since E2 and aldosterone appear to have opposing effects on endothelial function, we next explored whether E2 modulates MR transcriptional function in human vascular endothelial cells. Since ER expression is rapidly down-regulated in cultured ECs a human endothelial cell line (EAHy926 cells) was generated that stably expresses full length ERα. These ECs were transfected with the same MMTV MRE-luciferase reporter plasmid and treated with aldosterone (10 nM) in the absence or presence of E2 (10nM).

This concentration of aldosterone does not activate the GR (Figure 2.2C). As in human coronary ECs[13], aldosterone enhances MRE-Luciferase reporter activity in EAHy ECs (Figure 2.8A) although the magnitude is more modest than in HEK293 cells overexpressing the MR, likely due to the lower levels of endogenous MR expression in ECs. Aldosterone induction of luciferase activity is prevented by E2 supporting the concept that E2 interferes with aldosterone activation of endogenous MR transcriptional activity in human ECs. We next tested whether E2 modulates aldosterone-induction of a known MR target gene in ECs. We previously demonstrated that the cell adhesion molecular ICAM-1 is an MR transcriptional target gene in human coronary endothelial cells[13]. Treatment of EAHy926 ECs expressing ERα with aldosterone at 10 nM, a concentration found in patients with cardiovascular disease[154], increases expression of ICAM-1 mRNA. E2 (10 nM) prevents aldosterone induction of ICAM-1 mRNA in these cells (Figure 2.8B). These data offer evidence that E2 inhibits aldosterone-stimulated transcription in ECs and impacts mRNA abundance of a physiologically important MR target gene.

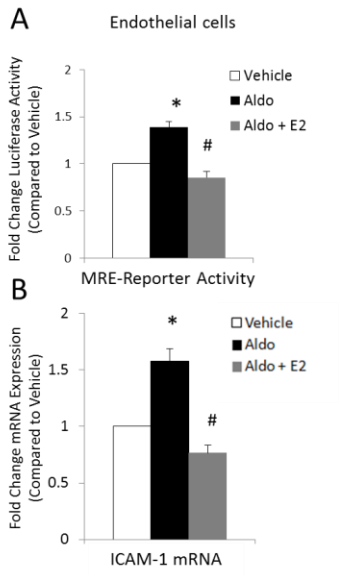

Figure 2.8. Estrogen inhibits aldosterone induction of MR transcriptional activity and of intercellular adhesion molecule-1 (ICAM-1) expression in endothelial cells. (A) Aldosterone activated MRE reporter activity is attenuated by E2 in ECs. Human endothelial cells (EAHy) stably expressing ERα were transfected with the MMTV MR-responsive reporter and luciferase activity was quantified in the presence of vehicle, 10 nM aldosterone (Aldosterone), or Aldosterone with 10 nM estradiol (Aldosterone + E2). (B) Estrogen prevents Aldosterone regulation of ICAM-1 in ECs. EAHy cells stably expressing ERα were treated with vehicle, Aldosterone, or Aldosterone + E2 for 18 hours and expression of intercellular adhesion molecule-1 (ICAM-1) mRNA was quantified by QRT-PCR. N=3 or 4 experiments. *$p<0.05$ versus vehicle; #$p<0.05$ versus Aldosterone.

Estrogen inhibits aldosterone induction of endothelial ICAM-1 protein expression and of leukocyte-EC adhesion

To explore the functional significance of estrogen inhibition of MR regulation of ICAM-1 mRNA expression, we assessed the effect of E2 on aldosterone induction of EC ICAM-1 protein expression and on aldosterone-stimulated immune cell adhesion to ECs. Aldosterone (10nM) increases ICAM-1 protein expression in ECs as quantified by immunoblotting with anti ICAM-1 antibody and this is prevented by E2

(10nM) (Figures 2.9A and 2.9B). We have previously demonstrated that aldosterone enhances leukocyte adhesion to human coronary ECs and that this requires ICAM-1[13]. To quantify leukocyte adhesion to ECs, endothelial monolayers were treated for 18 hours with 10nM aldosterone in the presence or absence of 10nM E2 and then incubated with fluorescent-labeled human monocytic cells (U937 cells). After washing away non-adherent leukocytes, adherent fluorescent cells were visualized (Figure 2.9C) and quantified (Figure 2.9D). Here we demonstrate that aldosterone enhances leukocyte adhesion to EAHy cells. Although E2 alone does not affect leukocyte adhesion to ERα-expressing EAHy cells, E2 prevents aldosterone-enhanced leukocyte-EC adhesion (Figure 2.9D). These data support the concept that by preventing aldosterone induction of MR target genes in ECs, estrogen may prevent the expression of genes that contribute to cardiovascular dysfunction and disease.

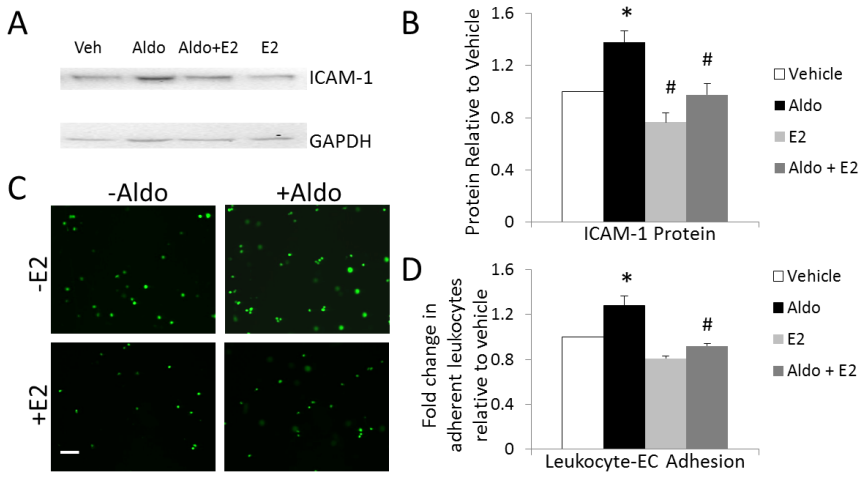

Figure 2.9. Estrogen inhibits aldosterone induction of ICAM-1 protein and of leukocyte adhesion to endothelial cells. Endothelial cells (EAHy) stably expressing ERα were treated with aldosterone (Aldosterone, 10nM), estrogen (E2, 10nM), or both for 18 hours and cell lysates were immunoblotted to quantify ICAM-1 protein (A and B) and incubated with fluorescently labeled human monocytic cells (U937) to quantify leukocyte-EC adhesion (C and D). (A) A representative ICAM-1 immunoblot is shown. (B) Quantification of ICAM-1 protein shows a significant increase with Aldosterone that is inhibited by E2. N=3. (C) Representative images of leukocyte adhesion assay. Scale bar=25μM. (D) Quantification of adherent fluorescent leukocytes reveals that E2 attenuates Aldosterone-stimulated leukocyte adhesion. N=4. *$p<0.05$ versus vehicle; #$p<0.05$ versus Aldosterone.

Methods

Reagents & cell lines

Aldosterone and E2 (Sigma Aldrich) were used as described[14;155] with DMSO (Sigma Aldrich) and ethanol (Fisher Scientific) controls, respectively. HEK293 cells (American Type Culture Collection (ATCC)) were maintained in phenol-containing DMEM (Gibco) with 10% fetal bovine serum (FBS, Atlantic Biologicals). HEK293 cells were switched to phenol-free 1% charcoal dextran stripped FBS (S-FBS) for 24 hours prior to any experimental treatment. For protein overexpression experiments, cells were transiently transfected with MR or ERα expression plasmids (described below) by PolyFect transfection reagent (Qiagen). Endothelial cell line EAhy926 cells (a human umbilical vein endothelial cell hybrid line, a kind gift of C.J. Edgell, University of North Carolina at Chapel Hill[158]) were grown in phenol-containing DME with 10% FBS and switched to 10% S-FBS prior to experimental treatment. EAhy926 cells were stably transfected with the pcDNA 3.1 ERα. Twenty-four hours after transfection, cells were placed in selective media with puromycin at 5ug/ml (Sigma) for 2 to 3 weeks. Single colonies were selected from 96-well plates that stably express moderate levels of ERα and maintained in the presence of 2 ug/ml puromycin. The expression of functional ERα was confirmed by ERα immunoblotting and ERE- luciferase reporter assays (data not shown).

Expression and Reporter Plasmids

The MR expression plasmid contains the full length human MR cDNA (a generous gift of R. Evans[17]) cloned into the CMX expression vector with an N-terminal hemagluttanin (HA) tag. The MR reporter plasmid contains the mouse mammary tumor virus (MMTV) long terminal repeat[159] cloned into the PGL2 luciferase reporter vector (Promega). The ERE reporter contains three copies of the Xenopus vitellogenin ERE24 (consensus sequence 5′-GGTCAnnnTGACC-3′) proximal to the thymidine kinase promoter driving expression of luciferase as described[160]. The construction of human full-length wild-type human ERα, ERα-S118A with an alanine for serine substitution at amino acid 118, pCMV3 ERα1-271, and pCMV3 ER 176-

595 have been described previously[160]. ERα 176-271 and 254-370 constructs were made by cloning PCR-derived ERα fragments into pC2 vector (Clontech) by *Eco*RI and *Sma*I sites, respectively. The His-tagged ERα plasmids were made by cloning ERα full-length and fragments into pet28a(+)(Novagen) by *Eco*RI and *Sma*I sites. The following plasmids were made by by QuikChange II XL Site–Directed Mutagenesis Kit (Stratagene). ER DBD mutants was made by alanine for cystine substitution in DBD domain at amino acid 220 and 227; ER KRR mutant was made by alanine for 231K, 233R and 234R substitution; ERα 1-263, 1-253, 1- 233 and 1-176 were made by insertion of a stop codon at ERα amino acids 263, 253, 233 and 176 respectively.

Transfections & luciferase assay

Luciferase reporter assays were performed as described[160] by co-transfection of plasmids for receptors (MR, ERα, ERα mutants), a plasmid for MR- or ERα-response element driving luciferase expression[159], and a beta-galactosidase plasmid to normalize for transfection efficiency. 24 hours later, cells were treated with aldosterone and/or E2 for 18 hours. Quantification of luciferase activity with Luciferase Assay Kit (Promega) was normalized to β-galactosidase activity assessed with Tropic accelerator (Applied Biosystems). Each treatment was carried out in triplicate and was performed in a minimum of three independent experiments.

Coimmunoprecipitation and Immunodetection

HEK293 cells were were co-transfected with the human HA-MR and ERα expression plasmids described above and switched to DME phenol red free medium with 10% S-FBS for 16 hours, then switched to serum free medium for 24 hours, followed by treatment with aldosterone, E2 or aldosterone plus E2 as indicated. Cells were harvested in lysis buffer (20 mM Tris-Cl, pH 7.5, 0.137 M NaCl, 2 mm EDTA, pH 7.4, 1% Triton, 10% glycerol, 25 mM glycerol phosphate, and in the presence of phenylmethylsulfonyl fluoride and protease inhibitor mixture). The coimmunoprecipitations were performed as described (41, 44) with the incubation of cell lysates with 5 micrograms of non-immune rabbit IgG or rabbit anti-HA antibody (Santa

Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4 °C. Protein G beads (Amersham Biosciences) were then added and a further incubation carried out at 4°C for 2 hours. The pellets obtained after centrifugation were washed five times with wash buffer (50 mM Tris, pH7.5, 7 mM MgCl2, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). The immunopellets were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and then probed with appropriate primary antibodies. Antibodies used for immunoblotting (Table 2.1) include rabbit polyclonal anti-ERα HC20 (Santa Cruz Biotechnology) and mouse monoclonal anti-MR (a generous gift from Dr. Celso Gomez-Sanchez[161]). Membranes were washed three times with wash buffer followed by incubation with anti-mouse or anti-rabbit– horseradish peroxidase secondary antibody (GE Healthcare UK LTD) and developed with ECL reagent (Amersham Biosciences).

Peptide/protein target	**Antigen sequence (if known)**	**Name of Antibody**	**Manufacturer, catalog #, and/or name of individual providing the antibody**	**Species raised in; monoclonal or polyclonal**	**Dilution used**
ERα	C-terminal	HC20	Santa Cruz Biotechnology	Rabbit polyclonal	1:1000
ERα	N-terminal	H184	Santa Cruz Biotechnology	Rabbit polyclonal	1:1000
MR			Dr. Celso Gomez-Sanchez (1)	Mouse monoclonal, Clone 6G1	1:100
Anti-mouse-HRP			GE Healthcare UK LTD		1:1000
Anti-rabbit-HRP			GE Healthcare UK LTD		1:1000
Flag-tagged M2	DYKDDDK		Sigma	Mouse monoclonal	1:500
Donkey anti-mouse cy3			Jackson ImmunoResearch		1:500

Table 2.1. Antibody table

His Pull-down Assay

His-ERα plasmids were transformed into *Escherichia coli* BL21(DE3) competent cells. His fusion protein expression was induced by 1mM Isopropyl β-D-1-thiogalactopyranoside for three hours. The fusion proteins were purified using Nickel-NTA agarose beads (Invitrogen). Expression of the fusions proteins was confirmed by SDS-PAGE and Coomassie Blue staining. The HEK293 cell lysates expressing human MR were incubated with 50 microliters of each His fusion protein beads. The samples were rocked at 4 °C overnight, washed 5 times to remove nonspecific binding, and boiled in SDS sample buffer. Associated proteins were resolved by SDS-PAGE and immunoblotted as described above.

Immunoflourescent staining

HEK293 cells were transfected with flag-tagged ERα, ERα mutants or ERα fragments. The cells were fixed in 3.7% paraformaldehyde for 10 minutes and permeabilized with 0.3%Triton X-100 for 15 minutes. After blocking with 10% donkey serum for 1 hour, immunostaining was performed as described[155;162] by incubating the cells with anti-Flag-tag (M2, Sigma) antibody for 1 hour (Table 2.1), washing three times with PBS followed by incubation with CY3-conjugated donkey anti-rabbit secondary Ab (1:1000) or Cy3-labeled donkey anti-mouse secondary Ab (1:1000) (Jackson ImmunoResearch). Cells were then washed three times with PBS and the nuclei were stained with DAPI (Sigma) for 15 min. Cells were analyzed using fluorescence microscopy.

Quantitative RT-PCR

RNA was isolated from endothelial cells, reverse transcribed, and quantitative RT-PCR was performed by methods that have been previously described[13;163]. *Ct* values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and mRNA levels in hormone-treated samples were expressed as a fold change relative to the expression level in vehicle-treated samples. Primer sequences were as follows:

ICAM-1, forward GGC CTC AGT CAG TGT GA and reverse AAC CCC ATT CAG CGT CA; GAPDH forward GAG CCA AAA GGG TCA TCA TCT CT and reverse GAG CCA AAA GGG TCA TCA TCT CT.

Leukocyte adhesion assay

EAhy926 cells stably expressing ERα were grown in DME with 10% S-FBS until 80% confluent and switched to serum free media for 24 hours. The cells were treated with aldosterone, E2, aldosterone plus E2 or vehicle for 18 hours. The leukocytes (U937 cell) were harvested, washed twice with PBS and labeled with BCECF-AM (2μM) (molecular probes, Eugene, OR) in PBS for 30 min at 37°C. The fluorescently labeled U937 cells were incubated with EAhy926 cells for 2 hours. Cells were gently washed with PBS 3 times to remove non-adherent cells. The adherent fluorescent cells were counted by a reader blinded to cell treatments as described[13].

Statistical Analysis

All values are reported as mean fold change compared to vehicle control ± standard error of the mean (SEM). Within group differences were assessed with one-factor ANOVA. Post-hoc comparisons were tested with the Student-Newman-Keuls test. $p<0.05$ was considered significant.

Chapter 3. Endothelial mineralocorticoid receptors differentially contribute to coronary and mesenteric vascular function without modulating blood pressure

Introduction

Ample clinical trial data reveal that mineralocorticoid receptor (MR) antagonist (MRA) drugs decrease blood pressure and improve survival in systolic heart failure[47-49;142]. It is well established that MRAs prevent the hormone aldosterone from activating renal MRs thereby decreasing sodium and water retention[164]. More recently, it has become clear that MR is expressed in tissues outside of the kidney including in the cardiovascular system. In the vasculature, MR is expressed in medial smooth muscle cells (SMCs)[14] and in intimal endothelial cells (ECs)[13]. SMCs and ECs function in concert to regulate arteriolar diameter, thereby globally controlling vascular resistance to contribute to systemic BP and locally modulating tissue blood flow[165]. Since the benefits of MRAs are disproportionately greater than their natriuretic properties, it has been postulated that some of their beneficial effects may be mediated by inhibition of vascular MR by mechanisms that are only beginning to be elucidated.

Recent studies in mice specifically deficient in MR in SMC have demonstrated that SMC-MR contributes directly to regulation of systemic BP and to vasoconstriction[166-168]. Despite substantial effort, our understanding of the specific role of EC-MR in BP control and vasoreactivity has been more elusive. In obese rats, MR inhibition improved coronary endothelial-dependent vasodilation and MR activation with aldosterone impairs coronary endothelial-dependent vasodilation in healthy rats[169]. Also in patients with congestive heart failure (CHF), MRA treatment improves brachial artery vasodilation[170] and in patients with diabetes, MRAs improve coronary flow reserve[84]. The studies suggest a role for endothelial MR in regulating vascular function in patients with cardiovascular disease or risk factors. Early studies exploring potential MR signaling mechanisms in cultured ECs revealed disparate effects of MR activation on endothelial nitric oxide synthase (eNOS) activity with reports of both MR-dependent inhibition and activation of eNOS activity or NO production (reviewed in[171;172]). Likewise, extensive experimentation has been performed in isolated vessels with variable effects of MR activation on vasoconstriction and vasorelaxation that may depend on the species, vascular bed, or experimental strategy employed[171].

Overall, the data support a role for vascular MR in vasomotor control yet the specific role of EC-MR is unclear from such studies in which the MR is activated or inhibited in SMCs and ECs simultaneously.

To examine the specific role of EC-MR in BP regulation and vasoreactivity, recent studies have utilized transgenic animals in which MR expression was modulated in the endothelium. Mice overexpressing human MR specifically in ECs have elevated BP, increased mesenteric myogenic tone and constriction, with no change in mesenteric endothelial-dependent vasodilation[63]. However, two groups recently deleted MR from ECs using a Tie2 promoter strategy revealing no difference in basal BP or vasoconstriction[64;65]. Using this model, Rickard et. al. demonstrated decreased aortic and mesenteric endothelial-dependent relaxation[64] while Schafer et. al. found no change in aortic endothelial function in healthy animals but protection from obesity-associated aortic endothelial dysfunction[65]. In addition to these conflicting results, these EC-MR knockout (EC-MR-KO) mouse models are complicated by MR deletion from leukocytes due to expression of the Tie2 promoter in bone marrow derived cells[65]. This has made interpretation of the specific role of EC-MR in this model difficult in light of the recent identification of a role for leukocytes in BP regulation[66] and a role for MR in modulating leukocyte function[67].

To clarify our understanding of the role of EC-MR in BP regulation and vasoreactivity, we generated an EC-MR-KO mouse with MR specifically deleted from ECs but with intact leukocyte MR using the VE-Cadherin (VE-Cad) promoter driving Cre-recombinase. Using this model, telemetry studies were performed to examine the role of EC-MR in BP regulation under normal conditions and in response to sodium loading/restriction and to RAAS activation. The role of EC-MR in endothelial-dependent relaxation and in vasoconstriction to multiple contractile agonists was explored in mesenteric and coronary arterioles to address the potential for EC-MR to differentially regulate vasoreactivity in distinct vascular beds. The contribution of EC-MR to arteriolar vasoconstriction and to the development of endothelial dysfunction after exposure to Angiotensin II hypertension was also examined in the model.

Results

A mouse model with MR specifically deleted from endothelial cells and intact in leukocytes

Mice with loxP sites flanking exons 5 and 6 of the MR gene (MRf/f) were bred with mice containing a Cre-recombinase transgene driven by the EC-specific VE-cadherin promoter (Cre+). EC-specific recombination of the MR gene was confirmed by PCR (Figure 3.1). Cultured ECs isolated from Cre+ mice showed complete MR DNA recombination while ECs from Cre- littermates showed no MR gene recombination (Figure 3.1A). DNA isolated from aorta, carotid artery, coronary artery, and mesenteric arterioles from Cre+ mice show recombination while vessels from Cre- mice do not (Figure 3.1B). DNA isolated from aorta, heart, and kidney of Cre+ mice revealed MR recombination consistent with the expected contribution of ECs in each tissue, while splenic leukocytes and lymph nodes contained only loxP MR, confirming lack of MR gene recombination in immune tissues and cells (Figure 3.1C). There was no recombination in tissues from Cre- mice. MR mRNA was also significantly decreased in primary ECs cultured from Cre+ compared to Cre- mice, while MR mRNA levels from splenic leukocytes were unchanged in Cre+ mice (Figure 3.1D). Thus MRf/f/Cre+ mice (EC-MR-KO) have recombined the MR gene specifically in ECs resulting in decreased MR expression in ECs compared to MRf/f/Cre- littermates (MR-intact) with intact MR DNA and RNA in immune cells (protein was not measured due to lack of effective mouse MR antibodies). The mice were born in Mendelian frequencies and showed no gross developmental differences supporting the conclusion that EC-MR is not necessary during embryologic development (Table 3.1).

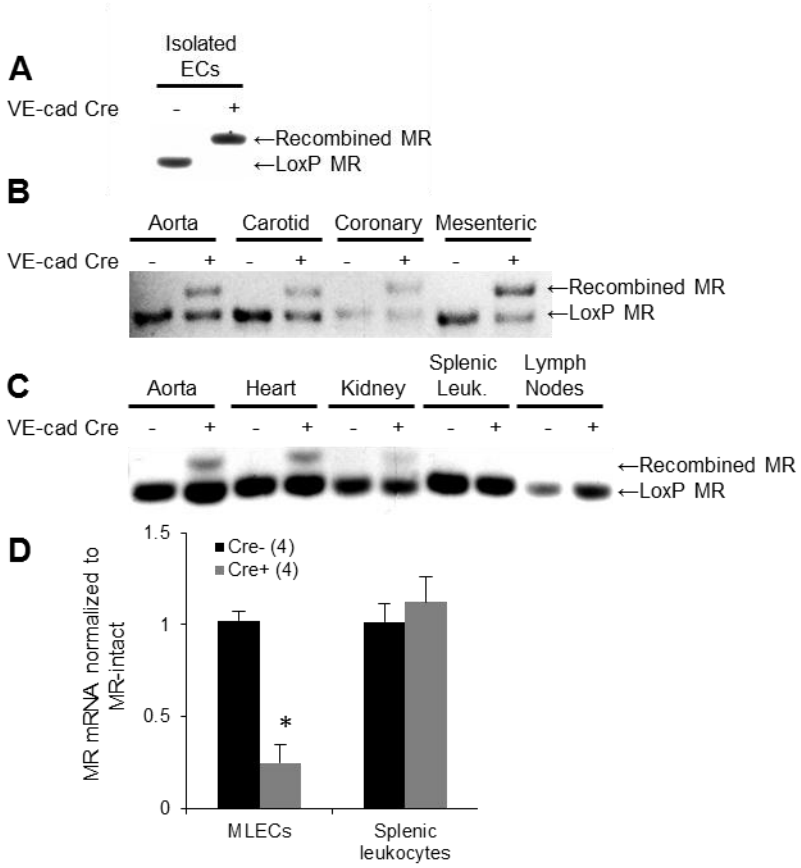

Figure 3.1. A mouse model with MR deleted specifically from EC. (A) The MR gene is recombined in vascular ECs. MR genomic DNA was amplified with primers specific for the LoxP MR or recombined MR in DNA isolated from whole aortas, aortas denuded of most ECs, and primary cultured mouse lung ECs. (B) Recombination occurs in VE-cad Cre expressing blood vessels from various tissues. (C) MR recombination occurs only in EC-containing tissues in VE-cad-Cre+ mice. MR genomic PCR was performed on DNA from Cre+ and Cre- aorta, heart, kidney, splenic leukocytes (Leuk), or lymph nodes. (D) MR mRNA is reduced in mouse lung ECs but not in leukocytes from EC-MR-KO mice. *$p<0.05$ versus MR-intact.

Parameter	**MR-intact**	**n**	**EC-MR-KO**	**n**
tibia length (mm)	18.14 (±0.1)	7	18.1 (±0.09)	7
body weight (g)	30.32 (±1.76)	7	29.2 (±0.68)	7
kidney weight (g)	0.17 (±0.01)	7	0.15 (±.02)	7
kidney length (mm)	9.97 (±0.25)	7	10.0 (±0.44)	7
serum aldosterone (pg/ml)	2.65 (±0.22)	4	3.04 (±0.58)	4
Electrolytes				
Normal sodium diet				
Serum				
sodium (mEq/L)	152 (±1.5)	3	148.3 (±1.85)	3
potassium (mEq/L)	5 (±0.32)	3	5.05 (±0.11)	3
creatinine (mg/dL)	0.23 (±0.12)	3	0.10 (±0.01)	3
blood urea nitrogen (mg/dL)	35 (±2.73)	3	31 (±4.62)	3
Urine				
sodium (mEq/L)	207.7 (±51.47)	3	226.13 (±42.38)	3
potassium (mEq/L)	>200	3	>200	3
creatinine (mg/dL)	122.13 (±42.78)	3	154.6 (±45.35)	3
urea nitrogen (mg/dL)	>250	3	>250	3
Low sodium diet				
Serum				
sodium (mEq/L)	149 (±0.56)	3	152 (±2.1)	3
potassium (mEq/L)	4.65 (±.68)	3	5.1 (±1.04)	3
creatinine (mg/dL)	0.1 (±9.8x10-18)	3	0.13 (±.03)	3
blood urea nitrogen (mg/dL)	40.8 (±2.9)	3	37.7 (±4.63)	3
Urine				
sodium (mEq/L)	21.65(±6)	3	17.08 (±1.7)	3
potassium (mEq/L)	>200	3	>200	3
creatinine (mg/dL)	122.13 (±42.78)	3	154.6 (±45.35)	3
urea nitrogen (mg/dL)	>250	3	>250	3
Food & water consumption				
Normal sodium diet				

Food (g)	3.1 (±0.86)	4	2.62 (±0.47)	4
Water (ml)	4.75 (±0.55)	4	4.88 (±1.0)	4
Low sodium diet				
Food (g)	2.3 (±0.29)	4	2.6 (±0.32)	4
Water (ml)	5.87 (±0.41)	4	5.97 (±0.35)	4

Table 3.1. Baseline characteristics of EC-MR-KO mice. Male EC-MR-KO mice and MR-intact littermates were used between the ages of 12 and 20 weeks. N for each genotype is indicated to the right. There are no significant differences in baseline characteristics between MR-intact and EC-MR-KO mice.

EC-MR does not contribute to basal, diurnal, salt sensitive, or RAAS-regulated blood pressure

This EC-specific MR KO mouse was next used to clarify the role of EC-MR in BP control. EC-MR-KO and MR-intact littermates were implanted with radiotelemetry devices for continuous ambulatory BP measurements. Specific deletion of EC-MR did not affect systolic, diastolic, or mean BP, heart rate, or activity level over the 24-hour cycle (Figure 3.2A and Figure 3.3). Systolic BP was unchanged from the age of 4 to 8 months in EC-MR-KO mice (Figure 3.2B). Since MR in the kidney plays a role in maintenance of BP in response to varying sodium conditions[173], salt sensitivity of BP was also assessed. Both genotypes showed modestly decreased BP when fed a low sodium diet and increased BP with high sodium diet, with no difference between the genotypes (Figure 3.2C). Serum and urine electrolytes and food and water consumption were also assessed on normal and on low sodium chow revealing no difference between EC-MR-KO mice and MR-intact littermates (Table 3.1). Fractional excretion of sodium ($FENa^+$) was not different between genotypes with the expected decline in $FENa^+$ on a low sodium diet, thereby confirming intact renal MR function in EC-MR-KO mice (Figure 3.2D). We next investigated whether EC-MR participates in the BP response to RAAS activation, a common contributor to hypertension[173]. BP increased to a similar extent in both EC-MR-KO and MR-intact mice in response to aldosterone infusion with a further increase with addition of 1% sodium in the drinking water. Likewise, both genotypes develop a

similar BP increase in response to Angiotensin II infusion that is further enhanced by addition of the eNOS inhibitor L-nitroarginine methyl ester (L-NAME) (Figures 3.2E and 3.2F).

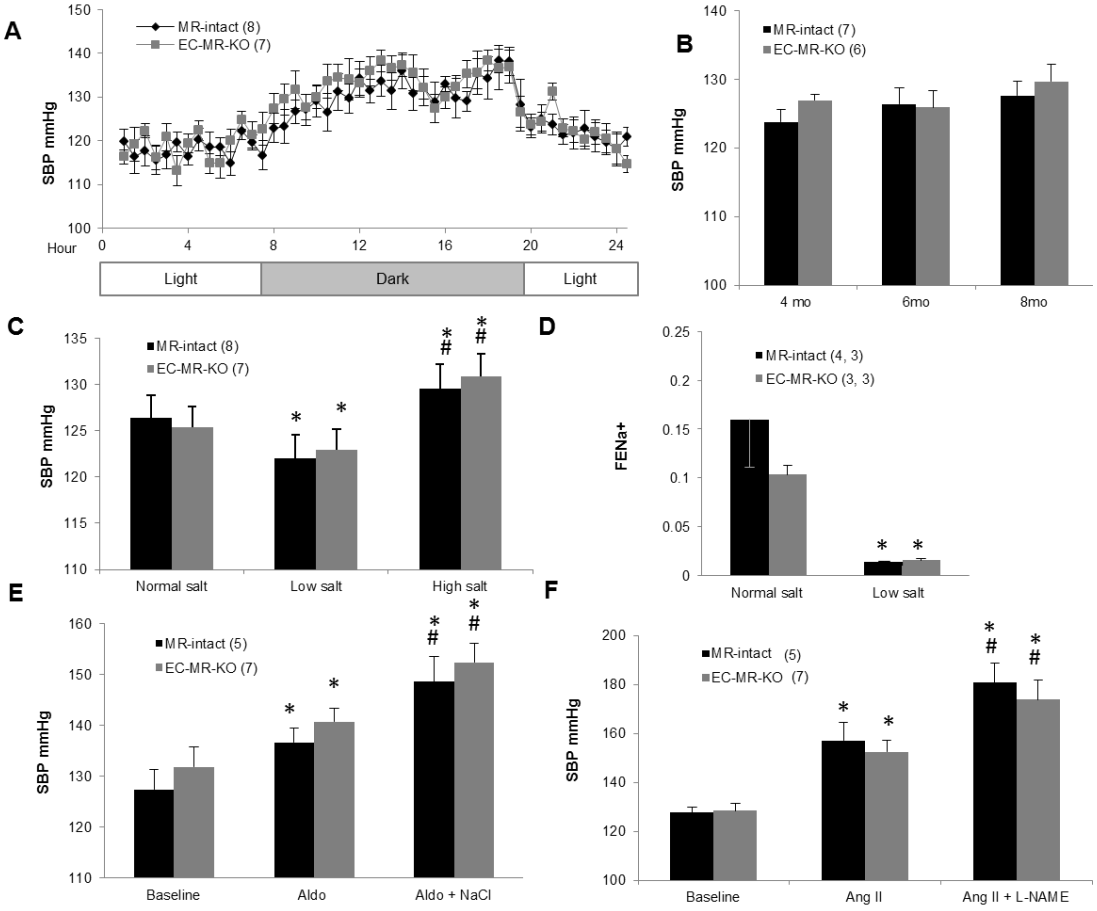

Figure 3.2. EC-MR does not contribute to basal, salt sensitive, angiotensin-enhanced, or aldosterone/salt-enhanced BP. (A) 24-hour ambulatory blood pressure in healthy 6-month old mice. (B) Average systolic blood pressure (SBP) at 4, 6 and 8 months of age in MR-intact and EC-MR-KO mice. (C) Average SBP on days 3-5 on normal, low, or high salt diets. *p<0.05 versus normal diet; #p<0.05 versus low salt diet. (D) FENa+ on normal and low-salt diet. *p<0.05 versus normal diet. (E) SBP on day 4 of aldosterone infusion; day 9 of aldosterone+1% NaCl in water. *p<0.05 versus baseline; #p<0.05 versus aldosterone alone. (F) BP on day 7 of Angiotensin II infusion; and on day 14 of Angiotensin II+L-NAME. *p<0.05 versus baseline; #p<0.05 versus Angiotensin II alone.

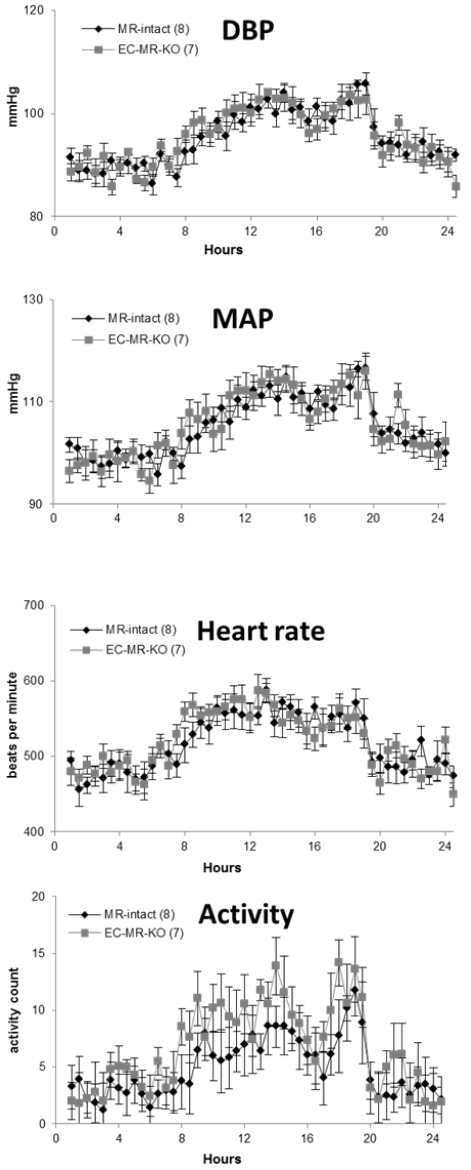

Figure 3.3. EC-MR deletion does not affect diastolic blood pressure (DBP), mean arterial pressure (MAP), heart rate or activity levels. Mice were 6 months of age for all measurements. Number of mice for each measurement are listed in parenthesis.

EC-MR deletion does not affect basal mesenteric or coronary arteriolar vasorelaxation

Since previous studies have reported conflicting findings regarding the role of EC-MR in vascular relaxation, we investigated the role of EC-MR in endothelium-independent relaxation to sodium nitroprusside (SNP) and endothelium-dependent relaxation to acetylcholine (Ach) in both mesenteric and

coronary arterioles. Mesenteric vasoreactivity contributes to systemic vascular resistance and regional blood flow and coronary vascular function is critical to coronary flow reserve, an important predictor of cardiovascular outcomes that was recently found to be modulated by MR antagonist treatment[84]. Wire myography revealed no difference in SNP or Ach response in mesenteric or coronary arterioles from EC-MR-KO mice compared to MR-intact littermates (Figure 3.4A-B). We conclude that EC-MR does not contribute to endothelium-dependent or endothelium-independent relaxation in unstressed mesenteric or coronary arterioles.

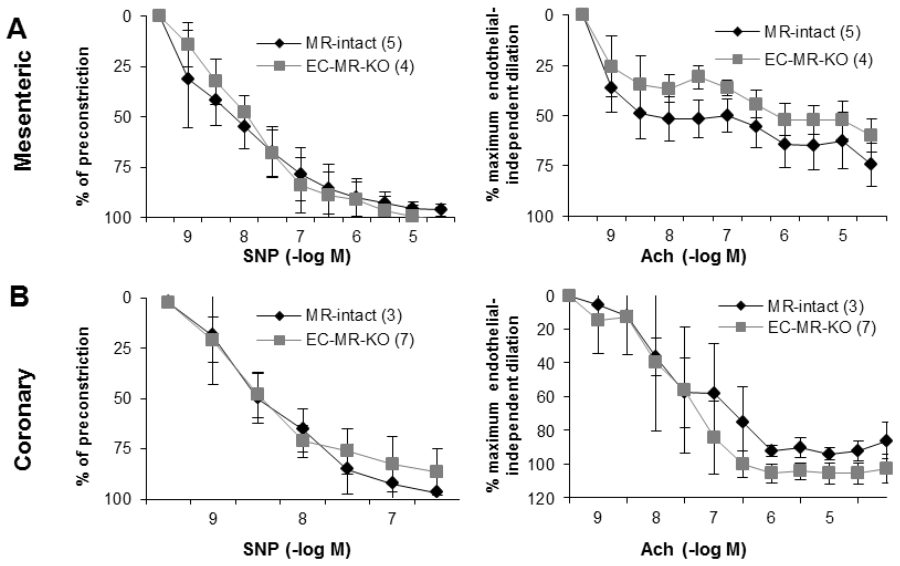

Figure 3.4. EC-MR does not contribute to mesenteric or coronary vasorelaxation. Sodium nitroprusside (SNP) and acetylcholine (Ach) dose-relaxation response curves following preconstriction to PE (10^{-6}M) in (A) mesenteric arterioles and (B) coronary arterioles. Number of animals per group is indicated in parentheses.

EC-MR modulates mesenteric endothelial-dependent vasorelaxation in the setting of angiotensin II-induced hypertension

We hypothesized that while EC-MR does not modulate vasorelaxation at baseline, it contributes to changes in vasorelaxation in response to hypertension. Mesenteric vasorelaxation was assessed in MR-intact and EC-MR-KO mice after 14 day infusion with a pressor dose of Angiotensin II as in Figure 3.2F. There was

no difference between genotypes in endothelial-independent vasorelaxation (Figure 3.5A) or in vasoconstriction to phenylephrine (PE) after Angiotensin II infusion (Figure 3.6) supporting that these SMC functions are unchanged by EC-MR deletion even after exposure to a hypertensive challenge. In assessing endothelial-dependent relaxation, we found different responses to Ach in MR-intact and EC-MR-KO mice following Angiotensin II exposure. EC-MR-KO vessels achieved a significantly greater maximal vasorelaxation compared to MR-intact controls after exposure to hypertension (Figure 3.5B). This enhancement was eliminated by pretreatment with L-NAME and indomethacin (Figure 3.5C), supporting that EC-MR-KO maintains maximum mesenteric Ach response after Angiotensin II-hypertension by enhancing NO and/or prostaglandin production (more likely NO since prostaglandins appear to contribute minimally to mesenteric relaxation in our preparation (data not shown)). After L-NAME and indomethacin pretreatment, EC-MR-KO vessels display significantly decreased response to 10-7M Ach compared to MR-intact vessels (Figure 3.5C). These data suggest that EC-MR in mesenteric arterioles contributes to a decline in NO-mediated vasorelaxation and an increase in endothelial derived hyperpolarizing factor (EDHF) after exposure to Angiotensin II hypertention.

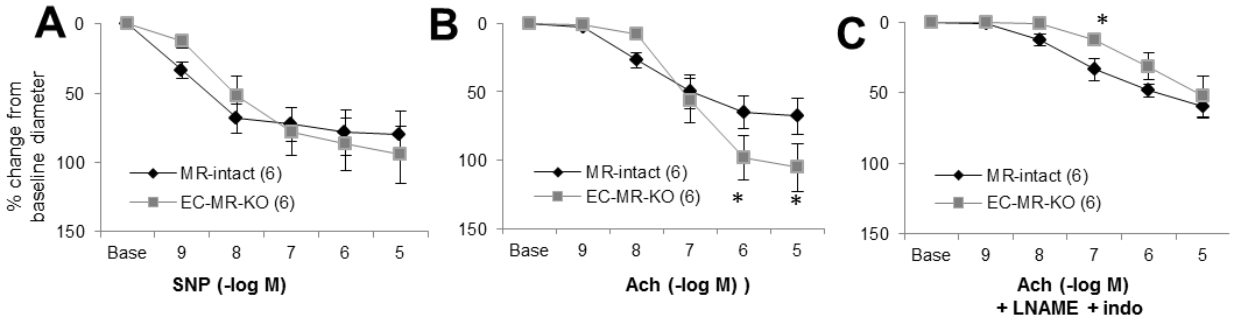

Figure 3.5. EC-MR deletion modulates mesenteric endothelial-dependent relaxation after exposure to Angiotensin II-induced hypertension. Mesenteric arterioles exposed to 14-day Angiotensin II infusion were pressurized to 70 mmHg, preconstricted to U46619 (0.1-0.3 µM), and the change in vessel diameter recorded in response to (A) Sodium nitroprusside (SNP), (B) Acetylcholine (Ach), and (C) Ach+L-NAME+indomethacin. *p<0.05 versus MR-intact. Number of animals per group is indicated in parentheses.

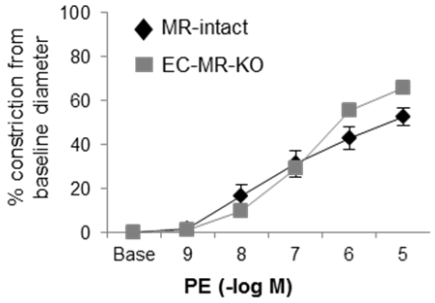

Figure 3.6. EC-MR deletion does not affect phenylephrine-induced vasoconstriction in mesenteric vessels exposed to 14 days of Angiotensin II. Mesenteric vessels from mice treated with 14 days Angiotensin II were pressurized to 70 mm Hg and the change in diameter to increasing concentrations of phenylephrine (PE) was quantified. N = 6/group.

Although coronary endothelium-dependent vasorelaxation to Ach was substantially impaired after exposure to Angiotensin II hypertension (Figure 3.7 compared to Figure 3.4B), it was not different between genotypes nor was there a difference in endothelial-independent relaxation to SNP. This finding supports the concept of vascular bed-specific differences in the role of EC-MR in hypertension-induced alterations in endothelial function.

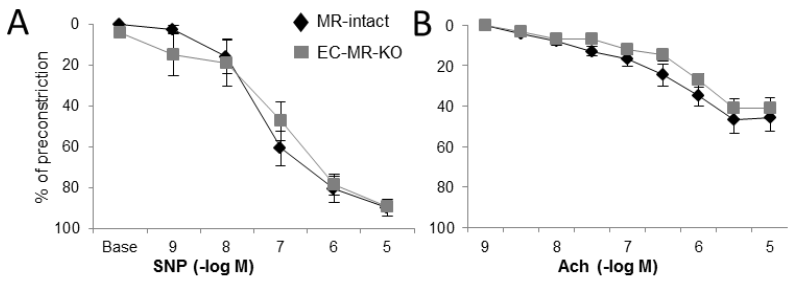

Figure 3.7. EC-MR deletion does not affect coronary arteriolar vasorelaxation following 14 days Ang II infusion. Coronary arteriolar relaxation to a dose-escalation of (A) Sodium nitroprusside (SNP) and to (B) acetylcholine (Ach). N = 6 mice/group.

EC-MR differentially modulates vasoconstriction in a vascular bed-specific fashion

Earlier studies demonstrated conflicting findings for the role of EC-MR in vasoconstriction using distinct models[171]. To clarify this, we assessed the specific role of EC-MR in the responses of mesenteric and coronary arterioles to a series of contractile agonists. There was no significant difference between EC-MR-KO and MR-intact controls in mesenteric arteriolar constriction to potassium chloride (KCl), endothelin-1, PE, or Angiotensin II (Figure 3.8A), with a decrease in constriction to the thromboxane agonist U46619 (42% decrease) in EC-MR-KO at a dose of $3x10^{-7}$M. Mesenteric myogenic constriction in response to increasing intraluminal pressure was also measured revealing no difference between EC-MR-KO and MR-intact mesenteric vessels in their myogenic tone (p=.478)(Figure 3.9), consistent with the lack of a difference in BP. Interestingly, coronary vessels from EC-MR-KO mice showed a decrease in constriction to U46619 (26% decrease at a dose of 10^{-6}M) and a pronounced decrease in constriction to endothelin-1 (49% decrease at 10^{-8}M) compared to vessels from MR-intact littermates (Figure 3.8B). These data support the concept that EC-MR differentially contributes to vasoconstriction depending on the vascular bed and on the contractile stimulus.

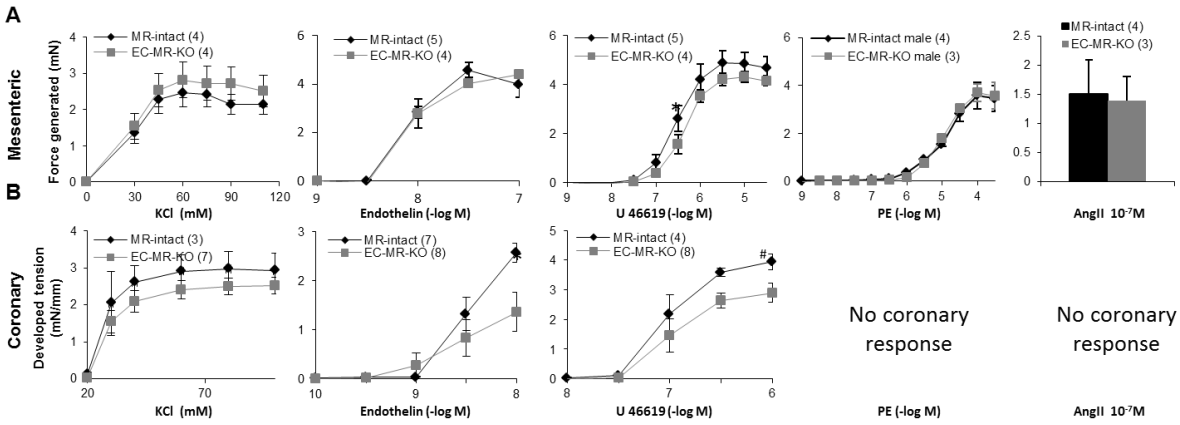

Figure 3.8. EC-MR differentially regulates vasoconstriction depending on the vascular bed and contractile agonist. Force generation was recorded by wire myography in response to a dose escalation in (A) mesenteric arterioles to KCl, endothelin-1, the thromboxane agonist U46619, PE, or a single dose of Angiotensin II; and in (B) coronary arterioles to KCl, endothelin-1, and U46619. No graph is included for PE and Angiotensin II as coronary arterioles do not respond to those agonists. *p<0.05, #p<0.005 versus EC-MR-KO. Number of animals per group is indicated in parentheses.

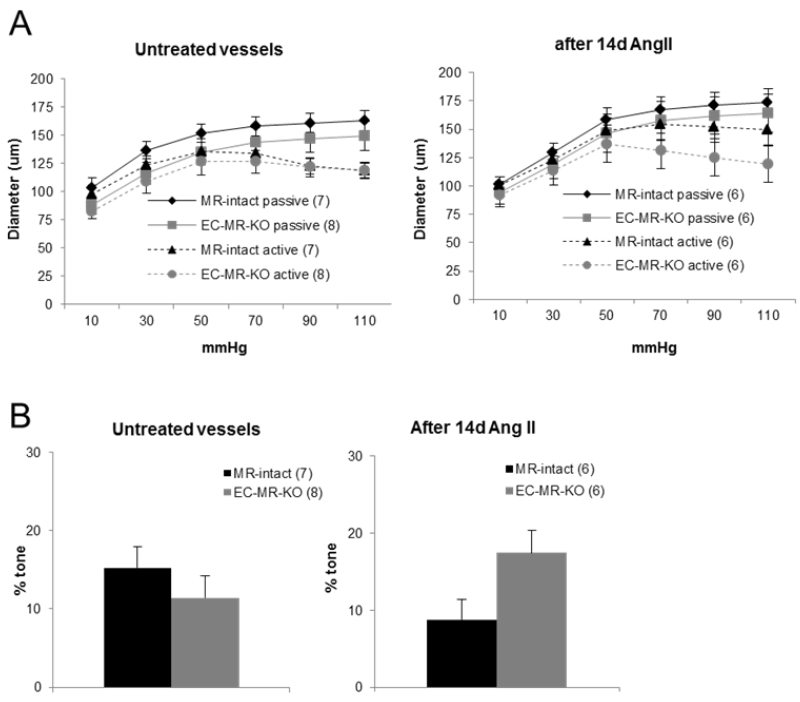

Figure 3.9. EC-MR deletion does not affect mesenteric myogenic tone response at baseline or after 14 day angiotensin II-infusion. N is indicated in parentheses. (A) Passive and active diameters of cannulated mesenteric arterioles from healthy mice (passive diameter, p=.273; active diameter, p=.478) or from mice infused with angiotensin II for 14 days (passive diameter, p=.594; active diameter, p=.346) over a range of intraluminal pressures. (B) Average spontaneous myogenic tone at 70 mm Hg is calculated as the percentage decrease in active lumen diameter from passive diameter for each mouse in A. There is no significant difference in spontaneous myogenic tone under either condition (untreated, p=.356; angiotensin II-treated, p=.057).

EC-MR contributes to coronary vasoconstriction to endothelin-1 and thromboxane after hypertension and to expression of endothelin-B receptor in coronary ECs

Coronary vasoconstriction to endothelin-1 and thromboxane was also measured after 14 day exposure to angiotensin II hypertension. The decreased coronary contractile response to endothelin-1 and thromboxane in EC-MR-KO mice persisted after hypertension and was significant over a greater range of agonist concentrations after exposure to 14 days of angiotensin II hypertension (Figure 3.10A and 3.10B). This was not due to differences in vasorelaxation after angiotensin II exposure (Figure 3.7). SMC L-type calcium

channels (LTCC) are required for vascular constriction however, single cell patch-clamp recordings of LTCC current density in freshly dispersed coronary and mesenteric SMC from EC-MR-KO and MR-intact controls revealed no difference in basal or activated LTCC function (Figure 3.11). We also considered whether EC-MR might contribute to expression of the receptors for endothelin and thromboxane in coronary ECs. Primary mouse cardiac endothelial cells were isolated from EC-MR-KO mice and MR-intact littermates and mRNA expression of the thromboxane receptor and of the vasodilatory endothelin-B receptor (ET_B) that is known to be expressed on ECs were measured, as well as expression of endothelin receptor A (ET_A), which is best known for its pro-constriction role on smooth muscle cells. ET_B expression was significantly increased (59%) in EC-MR-KO compared to MR-intact coronary ECs (Figure 3.10C), supporting the possibility that EC-MR contributes to coronary vasoconstriction in response to endothelin-1 by regulating coronary endothelial ET_B expression. ET_A expression and function on endothelial cells is less well characterized and is of unclear significance. Interestingly, expression of ET_A was decreased in coronary ECs from EC-MR-KO mice, suggesting some method of differential regulation may be occurring. Thromboxane receptor (TP) mRNA was unchanged in coronary ECs from EC-MR-KO mice suggesting a different mechanism for the enhanced coronary constriction to U46619 in mice deficient in EC-MR.

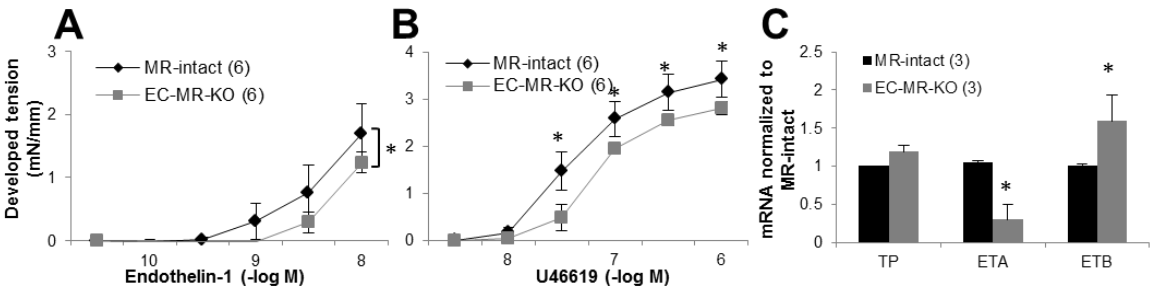

Figure 3.10. Decreased coronary vasoconstriction to endothelin-1 and U46619 in EC-MR-KO mice is accompanied by increased EC endothelin-B receptor mRNA. Coronary vasoconstriction was assessed after 14 day Angiotensin II infusion to a dose escalation of (A) endothelin-1 and (B) thromboxane agonist U46619. (C) Gene expression levels were assessed in RNA isolated from mouse cardiac endothelial cells (MCECs). TP=thromboxane receptor, ETB=endothelin-B receptor. *p<0.01; #p<0.05 versus MR-intact. N=3 experiments. Number of animals per group is indicated in parentheses.

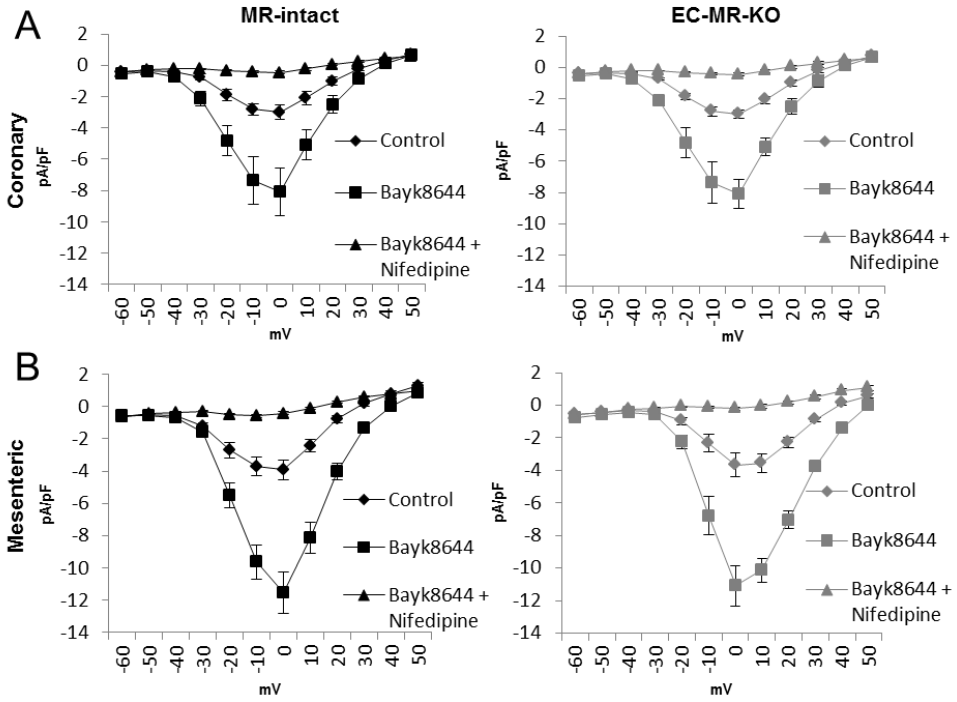

Figure 3.11. EC-MR deletion does not affect coronary or mesenteric smooth muscle cell L-type calcium channel function. L-type calcium channel (LTCC) current was measured in freshly dispersed smooth muscle cells from MR-intact and EC-MR-KO (A) coronary vessels (MR-intact: n=4 mice, 14 cells; EC-MR-KO: n=4 mice, 15 cells) and (B) mesenteric vessels. (MR-intact: n=7 mice, 31 cells; EC-MR-KO: n=9 mice, 37 cells). LTCC agonist: BayK8644 (2uM), LTCC inhibitor: nifedipine (1uM). There is no significant difference in LTCC current between MR-intact and EC-MR-KO SMCs.

Methods

Mice were handled in accordance with US National Institutes of Health standards and all procedures were approved by the Institutions Animal Care and Use Committees. All experiments were conducted on male mice aged 12-18 weeks on the C57Bl/6 background and compared EC-MR-KO mice to MR-intact littermate controls. Primers are listed in Table S1.

Generation of endothelial cell-specific mineralocorticoid receptor knockout mice

All mice were handled in accordance with US National Institutes of Health standards, and all procedures were approved by the Institutional Animal Care and Use Committees at the relevant institution. Mice lacking mineralocorticoid receptor (MR) specifically in endothelial cells (ECs) were generated by breeding mice with a loxP sites-flanking critical exons of the MR gene ($MR^{f/f}$)[167] with mice containing a Cre recombinase transgene driven by the EC-specific vascular endothelial (VE)-cadherin promoter (Cre+)[174]. All comparisons are made between male $MR^{f/f}$-VECad-Cre+ (EC-MR-KO) and $MR^{f/f}$-VECad-Cre- (MR-intact) littermates.

Isolation of primary endothelial cells

Mouse lung and coronary ECs were isolated as described previously[175]. In brief, hearts and lungs were harvested from 5-8 mice at 2-4 weeks of age. Organs were chopped finely with razor blades and digested in 2mg/ml of collagenase shaking at 37°C for 30 minutes. The digested organs were mechanically dissociated by triturating, filtered through a 70µm disposable cell strainer (Becton Dickinson Labware, Bedford, MA) and centrifuged at 500rpm for 8 minutes at 4°C. Sheep anti-rat-IgG Dynabeads were coated with anti-platelet endothelial cell adhesion molecule-1 (PECAM-1, Pharmingen) or anti-intercellular adhesion molecule 2 (ICAM-2) monoclonal antibody (Pharmingen)(2.5µ g antibody/$2x10^7$ beads) per the manufacturer's instructions. Beads were prepared and kept at 4°C ($4x10^8$ beads/ml of Dulbecco's phosphate-buffered saline (DPBS) with 0.1% FCS without sodium azide). The cell pellet was resuspended in cold DPBS and incubated with PECAM-1-coated beads (15µl/ml of cells) at room temperature for 10 minutes with end-over-end rotation. Magnetic isolation was used to recover the bead-bound cells. The recovered cells were washed with Dulbecco's modified eagle medium (DMEM containing 20% fetal calf serum (FCS) (DMEM-20%), resuspended in 10 ml of complete culture medium, (DMEM-20% supplemented with 100µg/ml heparin, 100µg/ml endothelial cell growth stimulant, nonessential amino acids, sodium pyruvate, L-glutamine, and antibiotics, at standard concentrations), and then plated in two gelatin-coated 65-mm dishes. After 48 hours in culture, media was removed and filtered through a Steriflip strainer. Media was replaced every two days with 50% conditioned, sterile-filtered medium plus 50% complete culture medium. When cells reached 75 to 85% confluence, they were detached with warm trypsin-ethylenediaminetetraacetic acid (Life technologies), pelleted and resuspended in DPBS and sorted again with ICAM-2-coated beads (15µl/ml of cells). Bead-bound cells were washed and plated in complete

culture medium and passaged further at a1:3 ratio. Monolayers of mouse lung endothelial cell (MLEC) isolates and mouse coronary endothelial cell (MCEC) isolates were used at passages 2 to 4 for experiments.

PCR analysis of Nr3c2 genomic DNA

Aorta, heart, kidney, and lymph nodes were isolated from EC-MR-KO and MR-intact littermates and frozen in liquid nitrogen. Splenic leukocytes were isolated by grinding of whole spleens through a 70μm filter followed by treatment with Red Blood Cell Lysing Buffer Hybri-Max (Sigma). DNA was extracted from each tissue, from cultured ECs, or isolated leukocytes with the DNeasy kit (Qiagen) and PCR was performed as described[167] using a combination of three primers listed in Supplemental Table 1.

Quantitative RT-PCR

RNA was extracted and reverse transcribed with an RNeasy mini kit (Qiagen), and quantitative RT-PCR was performed with gene-specific primers as previously described[163]. C_t values were normalized to β2-microglobulin (*B2MG*) and mRNA levels in EC-MR-KO samples were expressed as a percentage of those in MR-intact samples. Specific primers for quantitative RT-PCR are listed in Supplemental Table S1.

Blood pressure measurement by telemetry

For all surgical procedures, mice were anesthetized using 2.5% isofluorane gas. All blood pressure studies were performed using implantable blood pressure transmitters (Data Sciences International, TA11PA-C10) with n = 4–8 mice per group and blood pressure was recorded for 60 seconds every 30 min as previously described[167]. Mice were maintained on a 12 hour light-dark cycle with normal chow (0.3% NaCl; Harlan diet TD8604) and water available *ad libitum*. For salt challenges, mice with telemetric devices were fed a low-salt diet (0.02% NaCl; Harlan diet TD90228) or a high-salt diet (6% NaCl; Harlan diet TD90230) for 5 days with normal chow washout for 7 days in between, and blood pressure was averaged on days 3–5 of each diet. For aldosterone and salt administration, osmotic minipumps were implanted (Alzet) to infuse aldosterone (Sigma) at 240 μg kg^{-1} d^{-1}, for 2 weeks. BP was measured on Day 4. On Day 5, 1% NaCl was added to the drinking water followed by BP measurement on Day 9. For Angiotensin II administration, osmotic minipumps were implanted (Alzet) to infuse Angiotensin II (Sigma) at 800 ng kg^{-1} min^{-1} for 2 weeks. BP was measured on Day 7. After 1 week, L-NAME (0.2mg/ml, Sigma) was added to the drinking water and BP was measured on Day 14.

Serum and urine chemistries

Serum aldosterone was measured by radioimmunoassay (MP Biomedicals).Twenty-four–hour urine and simultaneous serum samples were collected from mice fed normal (0.3%) or low-salt chow in metabolic cages. Electrolytes were quantified (IDEXX Preclinical Services) and fractional excretion of sodium ($FENa^+$) was calculated: $FENa^+$ = (serum Cr × urine Na) / (serum Na × urine Cr) × 100.

Wire myography

Acetylcholine (Ach), indomethacin, L-N^G-nitroarginine methyl ester (L-NAME), phenylephrine (PE), and sodium nitroprusside (SNP) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Endothelin-1 and thromboxane A2 analog U46619 were purchased from Tocris (Bristol, UK). Chemical agents were dissolved in physiologic saline solution (PSS)/Kreb's buffers or dimethyl sulfoxide (only for U46619) based on solubility. Vessel segments (endothelium intact, 1.5–2 mm in length) were micro-dissected and mounted for isometric tension recordings (Danish Myo Technology, Model 610, Aarhus, Denmark) and data was analyzed using PowerLab software (AD Instruments) under conditions optimized for responsiveness of each vascular bed as described below. Ring segments were then equilibrated for 30 minutes. The vessels were maintained in PSS gassed (95% O_2 and 5% CO_2) to maintain pH (7.4) at 37°C.

Mesenteric myography

Mesenteric arterioles were brought to a resting tension of 2 millinewtons (mN) in tissue baths containing warmed (37 °C), aerated (95% O_2, 5% CO_2) PSS containing (in mM): 130 sodium chloride (NaCl), 4.7 potassium chloride (KCl), 1.17 magnesium sulfate ($MgSO_4$), 0.03 ethylenediaminetetraacetic acid (EDTA), 1.6 calcium chloride ($CaCl_2$), 14.9 sodium bicarbonate ($NaHCO_3$), 1.18 potassium phosphate (KH_2PO_4) and 5.5 glucose. Administration of 1 μM phenylephrine (PE) was used to test arteriolar viability, and the presence of intact endothelium was confirmed by acetylcholine (Ach, 1 μM)-induced relaxation of a half-maximal PE-induced contraction. Vasoconstrictor responses were assessed to PE (10^{-9}-10^{-4}M), endothelin-1(10^{-9}-10^{-7}M), U46619 (10^{-9}-10^{-5}M), KCl (30-120mM), and Angiotensin II (10^{-7}M). For relaxation studies, vessels were preconstricted with 1 uM PE before administration of Ach (10^{-9}-10^{-5}M) and SNP (10^{-9}-10^{-5}M).

Coronary myography

Coronary arterioles were brought to a tension equivalent to 90% of the diameter of the vessel at a transmural pressure of 90 mmHg as previously described[176] in tissue baths of warmed (37 °C), aerated (95% O2, 5% CO2), PSS containing (in mM): 118.99 NaCl; 4.69 KCl; 1.17 $MgSO_4$; 0.03 EDTA; 2.5 $CaCl_2$; 25 $NaHCO_3$; 1.18 KH_2PO_4; 5.5 glucose. Vessel viability was subsequently assessed by exposure to 80mM KCl. Vasoconstrictor responses were assessed to endothelin-1 (10^{-10}-10^{-8}M), U46619 (10^{-8}–10^{-6} M), and KCl (20-100mM). Preliminary studies showed that mouse coronary arterioles were not reactive to PE or Angiotensin II so full experiments were not performed with those agonists. For relaxation studies, vessels were preconstricted with U46619 (0.1-0.3 μM) before administration of Ach (10^{-9}-10^{-4}M) and SNP(10^{-9}-10^{-7}M). Coronary constriction data is shown as developed tension (force/mm) to account for variability of coronary vessel length.

Mesenteric vessel pressure myography

Mesenteric tissues were excised from anesthetized MR-intact or EC-MR-KO mice and transferred to a cooled chamber (4°C) containing dissection solution (in mM): NaCl 140; KCl 5.6; $MgSO_4$ 1; NaH_2PO_4 1.2; EDTA 0.02; sodium pyruvate 2; glucose 5; MOPS 3; albumin 0.1 mg/ml (USB Corporation, Cleveland, OH, USA); and pH adjusted to 7.3 with NaOH at room temperature. Segments (approximately 1–2 mm in length) of second-order mesenteric arterioles were micro-dissected (4°C), cannulated onto glass micropipettes, secured using 11-0 monofilament suture, and mounted in a 7 ml chamber of a cannulation stage as previously described[167]. The cannulated arterioles were filled with a modified Kreb's buffer containing (in mM): 111 NaCl; 4.9 KCl; 1.2 $MgSO_4$; 2.5 $CaCl_2$; 25.7 $NaHCO_3$; 1.2 KH_2PO_4; 11.5 glucose; 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); and pH adjusted to 7.3 with NaOH at room temperature. The cannulation stage was positioned on an inverted microscope and superfused (4 ml/min) with Kreb's buffer. Vessels were initially pressurized to 70 mmHg in the absence of luminal flow by connecting the inflow pipettes to a height-adjustable fluid reservoir. Length of the cannulated vessels was adjusted by stretching segment length with increasing intraluminal pressure (30–110 mmHg). Optimal length was determined by verifying that high intraluminal pressure (e.g. 110 mmHg) did not result in lateral bowing of the cannulated vessels. Vessels were warmed to 36–37°C during a 60-min equilibration period and allowed to develop spontaneous myogenic tone, which was usually 15–25 % of the maximal passive diameters in mice mesenteric arterioles. Changes in vessel diameter were observed and measured using

video microscopy and a video-based caliper system. Intraluminal pressures were elevated from 10 to 110 mmHg in 20 mmHg steps to test myogenic tone and obtain pressure-diameter relationships. Changes in diameter in response to (1) phenylephrine (1 nM–10 μM), (2) SNP (10 nM–10 μM), and (3) Ach (1 nM–10 μM) were monitored at 70 mmHg. After the assessment of SNP-dose response curves, phenylephrine (100 nM) was superfused as a pre-constrictor and Ach-concentration response curves were examined with the pre-constricted mesenteric arterioles in the absence or presence of L-NAME+indomethacin. SNP (5μM) was applied into the mesenteric arterioles after treatment with the two inhibitors to ensure that vascular smooth muscle cells function had not been compromised by the experimental procedure.

Electrophysiological recordings

Mouse left anterior descending artery (LAD) and mesenteric (2nd and 3rd order) arteriolar myocytes were isolated as previously described[177]. Whole cell currents of L-type calcium channel (LTCC) were recorded using a standard whole-cell patch clamp technique. Cells were superfused with physiological saline solution (PSS) containing (in mM): 138 tetraethylammonium chloride (TEA-Cl), 0.1 $CaCl_2$, 1 $MgCl_2$, 5 KCl, 10 HEPES, 10 Glucose, 20 barium chloride ($BaCl_{2)}$ pH 7.35 (Osm ~300 Osm/L). Pipette solution (in mM):120 CsCl, 10 TEA-Cl, 10 EGTA, 1$MgCl_2$, 15 HEPES, 5 Na_2-ATP, 0.5 Tris-GTP, 0.1 $CaCl_{2,}$ pH 7.2. LTCC I-V curves were obtained by using a holding potential of -70 mV, with step changes in potential from -60 to +50 mV, duration of 400 ms. Raw current values were normalized to cell capacitance and expressed as current density (pA pF−1). Reagents: BayK8644 (Sigma-Aldrich) and nifedipine (Sigma-Aldrich) were dissolved in 100% ethanol; final concentrations were Bay K 8644, 2 μM and nifedipine, 1 μM.

Statistics

Within-group differences were assessed with two-factor analysis of variance (ANOVA) or repeated-measures ANOVA (telemetry and mesenteric vessel contraction studies) with Student-Newman-Keuls post-test. $P<0.05$ was considered significant.

Chapter 4. Role of EC-MR in atherosclerosis

Introduction

MR activation is known to contribute to atherosclerosis development[115-118]. In healthy vasculature, ECs constitute a barrier to immune cell movement into the blood vessel. However in the early stages of atherosclerosis, ECs can become dysfunctional, characterized by a deficit in endothelial-dependent relaxation, and/or become activated, characterized by an upregulation of adhesion molecule expression on the EC surface. MR activation has previously been implicated in endothelial dysfunction in atherosclerosis[116] and MR antagonism ameliorates this dysfunction. Furthermore ECs play a critical role in the movement of leukocytes into the blood vessel. CD4+ T cells are a type of leukocyte that has been specifically demonstrated to contribute to atherosclerosis development (reviewed in[178]). Taken together with the knowledge that MR regulates expression of EC adhesion molecules[13], we hypothesized that EC-MR contributes to development of atherosclerosis through generation of endothelial dysfunction and endothelial activation.

	Male				Female			
	MR-intact (6)		EC-MR-KO (6)		MR-intact (6)		EC-MR-KO (5)	
	Mean	*±SE*	*Mean*	*±SE*	*Mean*	*±SE*	*Mean*	*±SE*
Pre-HFD								
Body weight	28.9	1.4	26.2*	0.6	19.9#	1.6	21.5#	0.7
SBP	107.1	4.9	120.9	2.8	110.1	7.7	120.7	7.6
DBP	80.2	3.4	89.6	3.7	74.9	4.2	84.5	5.1
Post-HFD								
Body weight	32.8	1.3	29.8*	1.0	22.8#	2.3	24.5#	0.7
Whole heart	0.15	0.00	0.14	0.00	0.12#	0.02	0.11#	0.00
Tibia length	17.9	0.2	17.7	0.1	17.6	0.2	17.6	0.1
Body weight/tibia length	1.84	0.06	1.69	0.05	1.29#	0.12	1.45#,^	0.03
Heart weight/tibia length	0.008	0.000	0.008	0.000	0.007*	0.001	0.007^^	0.000
Spleen	0.09	0.00	0.09	0.01	0.10	0.02	0.115^^^	0.01
Fasting blood glucose	258.0	17.4	301.0	8.3	265.7	38.1	249.5	21.1
SBP	109.5	4.3	115.6	8.6	118.2	3.3	120.6	3.5
DBP	76.6	5.1	79.0	8.1	78.6	2.4	84.6	2.1

Table 4.1. Baseline characteristics of EC-MR-KO/ApoE-/- mice. *p<.005 vs MR-intact; #p< .001 vs male; ^p<.005 vs MR-intact; ^^p<.01 vs MR-intact; ^^^p<.05 vs male

Results

Baseline characteristics of EC-MR-KO/ApoE-/- mice

To interrogate the role of EC-MR in atherosclerosis development, EC-MR-KO mice were bred with the atherogenic Apoliprotein E knockout (ApoE-/-) mice to create EC-MR-KO/ApoE-/- mice and MR-intact/ApoE-/- littermates. The ApoE-/- mouse is a commonly used genetic mouse model of atherosclerosis development. Apolipoprotein E (ApoE) circulates in the plasma in association with very low density lipoprotein (VLDL) and participates in hepatic clearance of plasma lipids[179]. Accordingly mice deficient in ApoE have been shown to be highly susceptible to atherosclerosis[180].

Baseline characteristics of EC-MR-KO/ApoE-/- mice were assessed prior to use in experiments. Pups were born in Mendelian frequencies without gross developmental defects. Male EC-MR-KO/ApoE-/- mice were modestly but significantly decreased in body weight at 18 weeks of age compared to MR-intact/ApoE-/- littermates (Table 4.1). There was no difference in body weight between female EC-MR-KO/ApoE-/- and MR-intact/ApoE-/- littermates. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were assessed using tail cuff plethysmography and there was no difference in SBP or DBP between EC-MR-KO/ApoE-/- mice and MR-intact/ApoE-/- littermates.

MR activation in the presence of high fat diet enhances leukocyte infiltration into the vasculature

We hypothesized that MR activation by aldosterone enhances leukocyte infiltration into the vasculature in the presence of cardiovascular risk factors. To test this hypothesis, three groups of mice were used: wild type (WT) fed normal diet, WT fed 4 weeks of high fat diet (HFD), and ApoE-/- fed 4 weeks of HFD. Two weeks into HFD diet exposure, each group was randomized to infusion of vehicle or aldosterone. After two weeks of infusion aortae were harvested and analyzed for CD4+ T cells by flow cytometry (Figure 4.1). In WT mice on normal diet, there was a very low basal number of CD4+ T cells and no change with aldosterone treatment. In WT mice on HFD, there was a significant increase in CD4+ T cell infiltration (2.21-fold increase with HFD), with a significant enhancement in aldosterone-treated WT mice on HFD (2.38-foldincrease in aldosterone-treated mice on HFD versus vehicle-treated mice on HFD). Finally, ApoE-/- mice on HFD showed a substantial increase in CD4+ T cells in the aorta (3.14-fold increase in

ApoE-/- mice on HFD versus WT mice on HFD), and aldosterone treatment further increased this infiltration (1.94-fold increase in aldosterone-treated mice versus vehicle-treated mice). Thus while MR activation in a healthy mouse does not affect CD4+ T cell infiltration, when exposed to high fat feeding and hyperlipidemia, EC-MR potentiates vascular T cell infiltration.

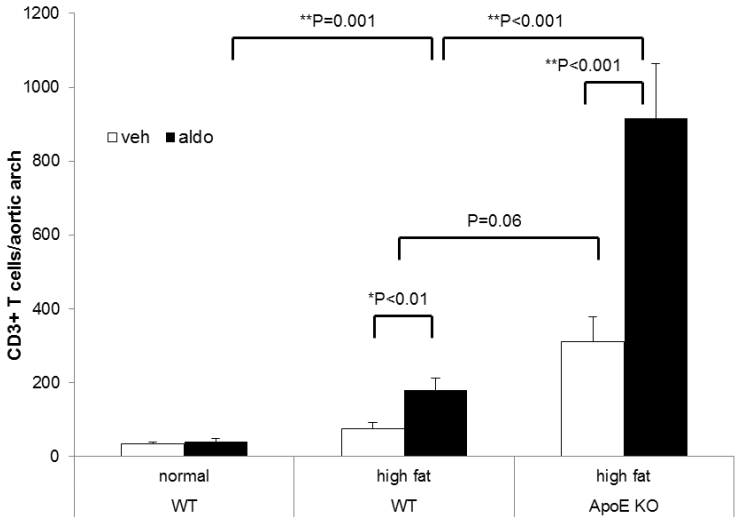

Figure 4.1. MR activation contributes to leukocyte infiltration into the vasculature in the presence of risk factors. CD3+ T cells in the aortic arch were quantified in wild-type (WT) mice on a normal diet, WT mice on a high fat diet (HFD), and atherogenic ApoE-/- mice on a HFD with 2-week vehicle or aldosterone infusion. N = 4/group

EC-MR deletion attenuates HFD-induced endothelial dysfunction in female mice as measured by pressure myography but not as measured by wire myography

Deficient endothelial-dependent vasodilation is an early finding in the development of atherosclerosis. We hypothesized that EC-MR plays a role in the endothelial dysfunction seen in the early stages of atherosclerosis. To test this hypothesis, female ApoE-/- were fed 4 weeks HFD and endothelial-dependent relaxation was first assessed via pressure myography. High fat feeding in ApoE-/- mice with intact EC-MR is associated with impaired endothelial-dependent relaxation. However, EC-MR-KO/ApoE-/- mice had significantly enhanced endothelial-dependent vasodilation compared to MR-intact ApoE-/- mice (Figure 4.2A). In contrast, a second cohort of EC-MR-KO/ApoE-/- females and MR-intact/ApoE-/- littermates

were fed four weeks HFD and then endothelial-dependent and-independent relaxation were assessed via wire myography. This experiment detected no difference in endothelial-dependent (Figure 4.2B) or –independent (Figure 4.2C) vasorelaxation in EC-MR-KO/ApoE-/- females compared to MR-intact/ApoE-/- littermates.

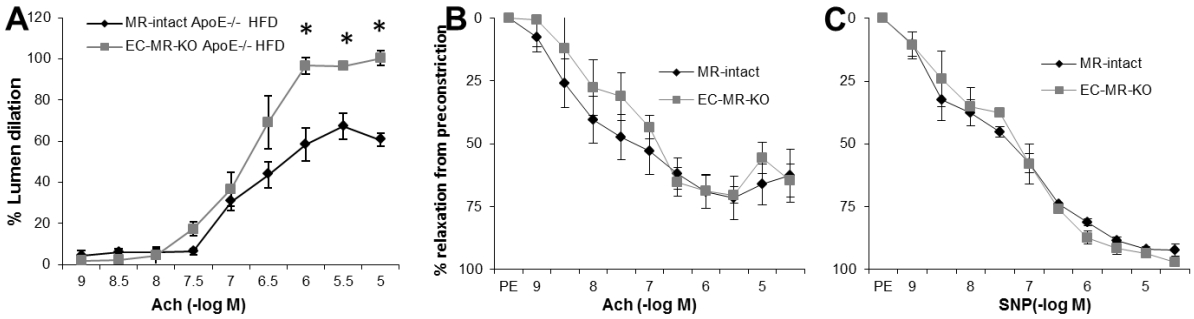

Figure 4.2. Role of EC-MR in endothelial-dependent relaxation in female ApoE-/- mice on HFD assessed using pressure and wire myography. Mesenteric vessels were preconstricted with phenylephrine and vessel diameter was determined in response to increasing concentrations of Ach. *p<.05 vs MR-intact ApoE-/-, n = 3 mice/group

Role of EC-MR in atherosclerotic plaque development in vivo

Male and female EC-MR-KO/ApoE-/- mice and MR-intact/ApoE-/- littermates were assessed for baseline characteristics including body weight and blood pressure prior to 4-week HFD and then following 4-week HFD. There was no difference in systolic blood pressure or diastolic blood pressure by genotype, sex, or dietary fat. Body weight significantly increased in male and female MR-intact/ApoE-/- and EC-MR-KO/ApoE-/- with HFD, and males weighed significantly more than females regardless of dietary fat intake (Table 4.1).

Atherosclerotic burden was evaluated in aortic valve leaflets from male and female MR-intact/ApoE-/- and EC-MR-KO/ApoE-/- mice following 4-week HFD. All results are preliminary due to low numbers of mice. There was a trend in male EC-MR-KO/ApoE-/- mice toward decreased atherosclerotic plaque size compared to male MR-intact/ApoE-/- littermates, while no trend was observed between female MR-intact/ApoE-/- and EC-MR-KO/ApoE-/- mice (Figure 4.3A). There was no suggestion of a difference in

plaque size between either MR-intact/ApoE-/- or EC-MR-KO/ApoE-/- male and female mice. Quantification of plaque lipid content by Oil Red O staining did not suggest differences according to EC-MR deletion or sex (Figure 4.3B).

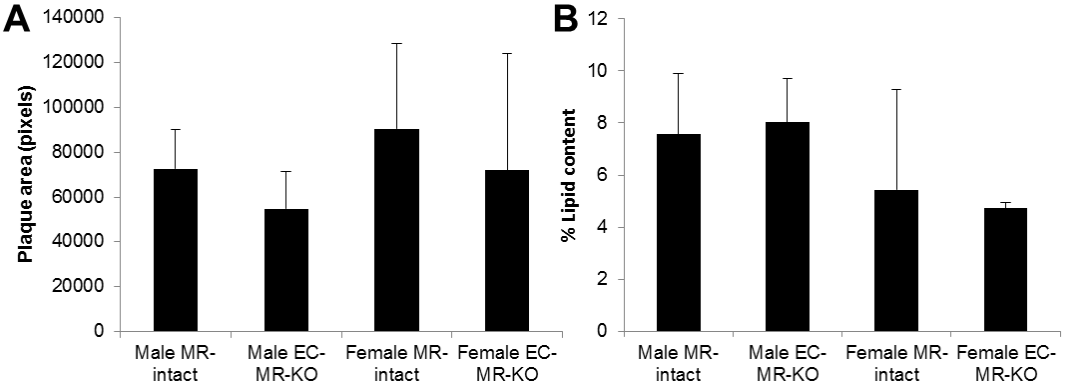

Figure 4.3. Preliminary data for plaque area and plaque lipid content with EC-MR deletion in a mouse model of atherosclerosis. (A) Plaque area by sex and genotype. (B) Plaque lipid content by % Oil Red O stain. N = 3 mice/group for male MR-intact, male EC-MR-KO, and female MR-intact; n = 2/group for female EC-MR-KO.

Methods

Mouse Atherosclerosis Model

All animals were handled in accordance with National Institutes of Health standards, and all procedures were approved by the Tufts Medical Center Institutional Animal Care and Use Committee. ApoE-/-mice (C57BL/6 background, Stock #002052) were purchased from Jackson Labs. EC-MR-KO/ApoE-/- double-knockout mice were generated by crossing EC-MR-KO mice with ApoE-/- mice. The resulting EC-MR-KO/ApoE-/- mice were used with MR-intact/ApoE-/- littermates as controls. Four-month-old male and female mice underwent tailcuff blood pressure measurements (as described below).

Following baseline blood pressure measurement, mice were placed on a proatherogenic high-fat diet (Harlan Teklad TD.88137). After 4 weeks, tail-cuff blood pressure measurements were repeated, and mice were euthanized for tissue collection.

Blood Pressure Measurements

Over the 5 days prior to pump implantation or animal euthanization, tail-cuff blood pressure measurements were performed using the Coda 6 System and software (Kent Scientific) by a 3-day training and measurement protocol that we have previously described and validated[143]. At the time of euthanization, animals were fasted for 4 hours, and blood was collected from the inferior vena cava. Animals were then perfused with phosphate-buffered saline (PBS) at physiological pressure for 1 to 2 minutes, and tissues were collected. The aortic valve and aortic arch were embedded in optimal cutting compound (OCT) and abdominal aortas fixed in 10% neutral buffered formalin as described[181;182]. Fasting blood glucose (Accu-Check) was measured immediately at the time of terminal tissue harvest.

Flow Cytometry

Aortic arches were stripped of adventitia and perivascular fat immediately after harvest and enzymatically digested as described[183]. Briefly, each arch was digested in 125 U/mL collagenase type XI, 60 U/mL hyaluronidase type I-s, 60 U/mL DNase1, and 450 U/mL collagenase type I (Sigma-Aldrich) in PBS containing 20 mmol/L HEPES at 37°C for 1 hour. Cells were filtered through a 70-lm filter to obtain a single cell suspension. Cells were then stained with APC-Cy7-conjugated anti-CD45.2 (BD Pharmingen), PE-Cy7-conjugated anti-CD3 (Biolegend), APC-conjugated CD11b (Biolegend), and FITC-conjugated CD107b (Biolegend) antibodies for 20 minutes at 4°C. Cells were analyzed on the FacsCantoII using FacsDiva software.

Mesenteric pressure myography

Mesenteric tissues were excised from anesthetized MR-intact or EC-MR-KO mice and transferred to a cooled chamber (4°C) containing dissection solution (in mM): NaCl 140; KCl 5.6; MgSO4 1; NaH2PO4 1.2; EDTA 0.02; sodium pyruvate 2; glucose 5; MOPS 3; albumin 0.1 mg/ml (USB Corporation, Cleveland, OH, USA); and pH adjusted to 7.3 with NaOH at room temperature. Segments (approximately 1–2 mm in length) of second-order mesenteric arterioles were micro-dissected (4°C), cannulated onto glass micropipettes, secured using 11-0 monofilament suture, and mounted in a 7 ml chamber of a cannulation stage as previously described1. The cannulated arterioles were filled with a modified Kreb's buffer

containing (in mM): 111 NaCl; 4.9 KCl; 1.2 MgSO4; 2.5 CaCl2; 25.7 NaHCO3; 1.2 KH2PO4; 11.5 glucose; 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); and pH adjusted to 7.3 with NaOH at room temperature. The cannulation stage was positioned on an inverted microscope and superfused (4 ml/min) with Kreb's buffer. Vessels were initially pressurized to 70 mmHg in the absence of luminal flow by connecting the inflow pipettes to a height-adjustable fluid reservoir. Length of the cannulated vessels was adjusted by stretching segment length with increasing intraluminal pressure (30–110 mmHg). Optimal length was determined by verifying that high intraluminal pressure (e.g. 110 mmHg) did not result in lateral bowing of the cannulated vessels. Vessels were warmed to 36–37°C during a 60-min equilibration period and allowed to develop spontaneous myogenic tone, which was usually 15–25 % of the maximal passive diameters in mice mesenteric arterioles. Changes in vessel diameter were observed and measured using video microscopy and a video-based caliper system. Intraluminal pressures were elevated from 10 to 110 mmHg in 20 mmHg steps to test myogenic tone and obtain pressure-diameter relationships. Phenylephrine (100 nM) was superfused as a pre-constrictor and changes in diameter in response to Ach (1 nM–10 μM) were monitored at 70 mmHg.

Immunohistochemistry

Cryosections of embedded aortic roots at the site where all 3 aortic valve leaflets could be visualized were taken at 6- to 10-lm intervals. Ten-micrometer sections were stained with Oil-Red O (ORO)to quantify lipids in the aortic root at the level of the aortic valve, as described[181;182]. Total pixels staining positive for ORO were normalized to overall plaque area to generate percent composition. Images were collected and analyzed by a treatment-blinded investigator using ImagePro 6.2 software (Media Cybernetics).

Chapter 5. Discussion

Transcriptional inhibition of endothelial MR by ERα

In Chapter 2, we demonstrated that: 1) ERα in the presence of estrogen prevents aldosterone stimulation of MR transcriptional activity; 2) ER and MR can associate as part of a common protein complex in cells; and 3) Estrogen inhibits aldosterone induction of the endothelial MR target gene ICAM-1 and prevents aldosterone-induced leukocyte-endothelial cell adhesion. The minimum portion of ERα required for MR transcriptional inhibition is the N-terminal amino acids 1-253 and the capacity of ERα to inhibit MR genomic function appears to require ERα localization to the nucleus and formation of a complex containing the two receptors. Inhibition of MR transcriptional activity does not require ERα to bind to DNA or to striatin nor is ligand-independent ERα activation necessary. We have previously shown that aldosterone-activation of EC MR induces ICAM-1 mRNA expression in human ECs by modulating ICAM-1 transcription and that enhanced endothelial ICAM-1 surface protein expression mediates aldosterone induction of leukocyte-EC adhesion[13]. Furthermore, aldosterone increases vascular leukocyte infiltration and atherosclerosis progression in animal models[119]. Here we demonstration that estrogen prevents aldosterone induction of endothelial ICAM-1 expression and of leukocyte-endothelial cell adhesion. These findings support the new concept that estrogen inhibition of MR-regulated transcription of pro-atherogenic genes in the endothelium could be a novel mechanism by which sex modulates cardiovascular risk.

While further studies are needed to clarify the detailed molecular mechanism(s) by which ER abrogates MR-mediated gene regulation, our findings are consistent with several possible mechanisms. First, the data support that the mechanism requires ERα to form a complex with MR in the nucleus, either through direct binding or indirectly via other proteins, and that this interaction blocks MR function as a transcription factor. This possibility is supported by our immunoprecipitation and nuclear localization experiments (Figures 2.5 and 2.6). However, while we identified ERα segment 1-253 as being the smallest portion able to inhibit MR transcriptional activity, the smaller segment 1-233 was able to complex with MR. The explanation for this discrepancy may come from our finding that nuclear localization of 1-233 was much weaker than 1-253, suggesting nuclear localization of ERα may also be critical for its interference with MR-mediated transcriptional activity. This mechanism appears to be distinct from the well characterized

dimerization interaction of the retinoid-X-receptor with non-steroid nuclear receptors including the thyroid, retinoid and vitamin D receptors (reviewed in[184]) since this does not require DNA binding by the estrogen receptor. Once ER forms a complex with MR in the nucleus, multiple potential mechanisms are consistent with the data. ER may prevent MR from interacting with a cofactor that is required for aldosterone-induced transcriptional activation but is not needed for ER transcriptional activation. This is consistent with the finding that MR does not mutually inhibit transcriptional activation of ER (Figure 2.1B). Recently, post translational modification of the MR by phosphorylation has been shown to modulate MR transcriptional function in specific renal cell types[26]. Thus another possibility could be that complexing with ER in the nucleus facilitates MR modifications that alter its transcriptional function. The N-terminus of MR has also been shown to interact with the ligand-binding C-terminal domain of MR and this "N-C interaction" is enhanced by aldosterone and modulates MR transcriptional function[185;186]. Although we have not investigated which domain of MR mediates complex formation with ER, since the ERα N-terminus appears to be required to modulate MR function, it is possible that the ERα N-terminus effects the MR N-C interaction to modulate MR transcriptional function. Further studies are needed to differentiate between these mechanisms.

There are important limitations of this study that should be considered. The initial in vitro studies exploring interactions between ER and MR transcriptional function utilize a transformed human embryonic kidney cell line (HEK293 cells) with overexpressed ER and MR proteins and a transfected supercoiled plasmid containing the MMTV MR-responsive element as a transcriptional reporter. This system, although highly artificial, was chosen because these HEK293 cells lack endogenous ER, MR, and GR and hence expression of receptors and ER mutants could be carefully controlled to specifically and independently examine transcriptional function of the ER and the MR (in the absence of GR). The MMTV reporter was used because much of what is known about MR transcriptional function has been determined using this promoter[187;188] and there are no vascular MR target genes for which the MR DNA-binding site has been determined. The lack of known vascular MR target gene DNA binding sites and an adequate MR antibody for chromatin immunoprecipitation (ChIP) has prevented the use of more modern methods to confirm endogenous MR binding to specific target promoters within intact chromatin[189]. To address these limitations, we confirmed the MMTV reporter studies in more physiologically relevant vascular endothelial

cells with endogenously expressed MR and also examined the effects of estrogen on aldosterone regulation of an endogenous vascular MR target gene in the context of intact chromatin. Another important limitation is that ER expression is rapidly down-regulated in cultured cells thus the ER in all of these studies is exogenously expressed. Future studies in whole vessels or in vivo will be needed to interrogate this novel mechanism in the setting of physiological levels of MR and ER. Whether such a mechanism also applies to other combinations of steroid receptors also remains to be determined. Other considerations limit the interpretation of the effects of E2 on aldosterone regulation of endogenous ICAM-1 in human ECs. In this system, one cannot exclude an independent effect of ER on ICAM-1 transcription. This is less likely since estrogen alone did not effect ICAM-1 expression or leukocyte adhesion but rather, estrogen attenuated the effects of aldosterone on ICAM-1 expression and function (Figure 2.9C and 2.9D). Also, since ICAM-1 mRNA induction by aldosterone is very modest after short term treatment (only 20% increase after 3 hours[13]) the studies here were performed after 18 hours of aldosterone treatment leaving open the possibility of secondary effects beyond transcriptional activation. We have previously demonstrated that aldosterone-induction of ICAM-1 mRNA in human coronary endothelial cells is completely inhibited by Actinomycin D even after 24 hours[13] supporting a transcriptional mechanism. However, that does not rule out the possibility that ER might also interfere with longer term effects of the MR on ICAM-1 regulation.

Despite these limitations, the findings presented here are important in the larger context of substantial evidence for opposing roles for E2-activated ER and aldosterone-activated MR in human cardiovascular disease. The decreased incidence of cardiovascular disease in premenopausal women compared to age-match men that equalizes after menopause is well-known[190] yet the mechanism is still not clear. In addition, serum aldosterone levels are lower specifically in premenopausal women compared to age-matched men[191-193]. Conversely, elevated serum aldosterone levels correlate with increased incidence of cardiovascular disease and poor outcomes[114;141]. Sex may also be an important factor in the cardiovascular response to aldosterone, as suggested by clinical evidence that serum aldosterone directly correlates with left ventricular remodeling in women while no such correlation was found in men[194]. The finding that higher serum aldosterone levels correlate not only with atherosclerotic progression but specifically with endothelial dysfunction in humans more specifically implicates aldosterone activation of endothelial MR in cardiovascular disease[149].

Experimental studies in animal models of cardiovascular disease further support the concept that estrogen and aldosterone act in opposition in the cardiovascular system. While E2 treatment inhibits the pathologic vascular remodeling response to carotid injury in mice[195], aldosterone infusion exacerbates remodeling in that same vascular injury model[143]. Similarly in the apolipoprotein E knockout mouse (ApoE-KO) model of atherosclerosis, E2 treatment decreases atherosclerotic plaque size and immune cell infiltration in the plaques while aldosterone treatment increases plaque size and vascular inflammation[119;144]. Moreover, treatment with ER agonists attenuate MR-mediated hypertension and vascular fibrosis in rats infused with aldosterone[196]. Estrogen via ERα has also been found to inhibit angiotensin II-induced hypertension and cardiac fibrosis[197]. As many of the cardiovascular consequences of angiotensin II are mediated by aldosterone and MR[198], these data could also provide a mechanism for the interaction between E2 and angiotensin II. One important limitation of many of these animal studies is that they were performed only in one sex and thus additional studies are required to directly compare the effects of E2 and aldosterone in males versus females. However, one unifying aspect of these diverse cardiovascular disease models is the disruption of endothelial cell function, whether through mechanical injury of the endothelium in the carotid injury models, oxidative stress in the angiotensin II hypertension model, or inflammation in the atherosclerosis model. The ubiquitous involvement of the endothelium in models of cardiovascular disease that are differentially affected by aldosterone and E2 implicate the endothelium as a tissue in which E2 modulation of MR function may have profound clinical implications. Indeed, aldosterone-induced vascular inflammation is thought to be mediated at least in part by upregulation of EC ICAM-1[13]. E2 inhibition of aldosterone-induction of EC ICAM-1 expression and leukocyte adhesion would be expected to decrease plaque inflammation thereby preventing plaque rupture, the cause of most heart attacks and strokes[172]. Thus, these findings may represent an important new mechanism explaining the lower incidence of cardiovascular ischemic events in premenopausal women.

Future directions

In conclusion, the data in chapter 2 identifies a novel mechanism in which E2 signaling through ERα inhibits transcriptional regulation by aldosterone and the MR in the vascular endothelium. These data demonstrate an interaction between ER and MR that may offer critical insight into the mechanism of

gender differences in cardiovascular pathologies as well as potentially modulating endothelial pathways beyond those explored in this study. Further work will be required to determine if this interaction is relevant in animal models of cardiovascular disease and in human pathology. Ultimately, greater understanding of this pathway may offer new therapeutic opportunities for the prevention and treatment of cardiovascular disease in both men and women.

Endothelial mineralocorticoid receptors differentially contribute to coronary and mesenteric vascular function without modulating blood pressure

In Chapter 3, we developed the first truly EC-specific MR-KO mouse model with intact immune cell MR to clarify the specific role of EC-MR in BP regulation and vascular function. Using this model, we demonstrated that: 1) EC-MR deletion does not alter renal sodium handling, basal BP, or BP after diverse hypertensive stimuli; 2) EC-MR does not contribute to basal endothelium-dependent relaxation in mesenteric or coronary arterioles but modulated mesenteric (not coronary) endothelial function after exposure to Angiotensin II-hypertension; 3) EC-MR deletion improves mesenteric endothelial function after hypertension in an NO- or prostacyclin-dependent manner; 4) EC-MR differentially regulates vasoconstriction in distinct vascular beds, enhancing the responsiveness of coronary arterioles to endothelin-1 and thromboxane both at baseline and after angiotensin II hypertension. Overall, these studies provide substantial new data supporting that EC-MR does not contribute to BP regulation in mice under basal and most experimental conditions. Rather, these data uncover a role for EC-MR in regulation of vasoconstriction and in modulation of endothelial function after hypertension that is vascular bed-specific and may explain some of the seemingly conflicting findings in prior studies. These results also support new therapeutic opportunities for targeting of pathways critical to maintenance of tissue-specific perfusion, particularly to the heart, in the setting of cardiovascular disease.

This study provides substantial clarification of the role of EC-MR in BP control in the context of the existing literature. It is quite clear that MR in the kidney contributes to BP regulation and it has recently been demonstrated that MR in SMC also contributes to BP regulation by modulating vascular tone[166;167]. This study provides the first data using telemetry as the gold standard approach to BP measurement in a model in which MR is specifically deleted from ECs. From these data we conclude that EC-MR does not

contribute to systolic, diastolic, mean, diurnal, or salt-sensitive hypertension. Furthermore, EC-MR deletion does not alter the response to typical hypertensive stimuli including mineralocorticoid excess/salt or Angiotensin II+/-L-NAME. This is consistent with the previously published Tie2-Cre EC-MR-KO model in which BP was measured by tail cuff and was not different in Cre+ animals at baseline or after uninephrectomy/DOCA/salt treatment[64]. The finding that EC-MR deletion does not modulate BP contrasts with the transgenic mouse overexpressing human endothelial MR which has substantially elevated BP[63]. While it is possible that the hypertension in the overexpression model is due to supraphysiologic levels of MR expression or to a functional difference between human and mouse MR, it is also possible that there are conditions in which MR is substantially upregulated in the endothelium (such as aging or heart failure) in which EC-MR might play an enhanced role in BP control and further studies are needed to explore those possibilities.

This study is also important because the previous EC-MR-KO model using the Tie2 promoter to control expression of Cre-recombinase also triggers recombination of MR in immune cells. This relates to the timeline of expression of Tie2 compared to the VE-Cadherin promoter used in our study. While Tie2 is expressed in mouse development as early as E6.5[199] in hemangioblasts that will ultimately differntitae into both hematopoietic cells and endothelial cells, embryonic VE-Cadherin expression occurs around E9.5[174]. Therefore prior to the differentiation of hematopoietic and endothelial cell lineages, Cre-recombinase expressed under control of the Tie2 promoter will cause recombination of MR in both immune cell populations including T cells, B cells, and monocytes as well as in endothelial cells. In contrast, Alva et al demonstrated Cre expression in a minority of hematopoietic cells and we have found no evidence of recombination in leukocytes. Thus this technical approach to generating an endothelial cell-specific MR KO mouse benefits from the increased specificity of an endothelial cell promoter that is expressed later in development than Tie2.

These results also add to a growing body of literature supporting the idea that EC-MR may have a minimal (or even a slight vasodilatory) role in vessels under basal conditions (reviewed in [171]) but that EC-MR participates in the development of endothelial dysfunction in response to cardiac risk factors including obesity[65] and now also after exposure to hypertension[200]. We demonstrate no difference in basal mesenteric

or coronary vasodilation in EC-MR-KO mice and Schaefer et. al. reported similar findings in the aorta of Tie2-MR-KO mice[65]. This contrasts with the Rickard et. al. study that found decreased aortic and mesenteric endothelial-dependent relaxation in Tie2-MR-KO mice[64]. The difference in mesenteric response in our model and the Rickard study could be due to the use of a model driving Cre expression with the Tie2 versus the VE-cadherin promoter with immune cell MR contributing to mesenteric vascular function in the Tie2-driven model or to technical variations in how the vessel studies were performed. However, after exposure to risk factors, studies consistently show that EC-MR contributes to changes in endothelial function induced by obesity[65] and now also in angiotensin II-induced hypertension. The increased maximum Ach response in EC-MR-KO mesenteric vessels after hypertension was lost with pretreatment with L-NAME and indomethacin suggesting that EC-MR activation in the setting of angiotensin II-induced hypertension may inhibit the production of NO or prostacyclin (more likely NO since prostacyclin contributes minimally to mesenteric relaxation). The decrease in the EDHF contribution to relaxation in EC-MR-KO vessels (Figure 3.5C) is consistent with studies supporting a role for EC-MR in regulation of the vasodilatory potassium channel SKCa, a component of EDHF[201]. Further research is needed to explore the mechanistic link between angiotensin II exposure and EC-MR regulation of NO production and EDHF. It is also important to note that the dose of angiotensin II that resulted in endothelial dysfunction in MR-intact vessels from which EC-MR-KO vessels were protected generated a blood pressure rise in MR-intact and EC-MR-KO mice that was not different between genotypes. Although the global eNOS KO mice are hypertensive, regional endothelial dysfunction is generally not associated with changes in systemic blood pressure but rather reflects endothelial damage and is an independent risk factor for cardiovascular disease[64;78;79;83;202]. Future studies are needed to examine the role of EC-MR in endothelial function in the setting of other hypertensive stimuli including aldosterone plus high salt.

Although the specific role of EC-MR is difficult to determine in humans, clinical data support that MR activation may play a greater role in endothelial function in patients with high cardiovascular risk including those with hypertension, diabetes, and heart failure[83;84;170;203] rather than in those with a healthy vasculature[204]. Thus, MR antagonism may provide clinical benefit in the setting of cardiovascular risk factors such as hypertension or obesity by interfering with the development of endothelial dysfunction, an important early step in the development of atherosclerosis that also has negative prognostic implications.

Our study additionally supports the concept that EC-MR contributes to vasoconstriction in a manner that depends on the vascular bed examined and on the contractile agonist tested. This finding may explain what appear to be conflicting results in the literature. For example, the EC-MR-overexpressing mouse showed enhanced mesenteric vasoconstriction to multiple contractile agonists including KCl, endothelin-1, U46619, PE and angiotensin II[63] while the Tie2-MR-KO mouse showed no difference in vasoconstriction in the aorta[65] potentially due to vascular bed differences. However, both our study and that of Rickard et. al. found that EC-MR-deletion had no effect on mesenteric constriction to the same agonists. Although it was recently demonstrated that MR antagonists reverse coronary vascular dysfunction in a rat model of obesity[169], this is the first study to explore the specific role of EC-MR in coronary function. Our data reveal that EC-MR deletion decreased the coronary vasoconstrictor response to endothelin-1 and thromboxane, both at baseline and after exposure to AngII hypertension. Comparisons between the coronary response with and without AngII (Figure 3.8 versus Figure 3.10) cannot be made as the studies were not performed simultaneously and the necessary controls were not included to make such comparisons (no vehicle pumps in Figure 3.8). These data demonstrate that EC-MR contributes to coronary vasoconstriction to endothelin and thromboxane and further supports vascular bed-specific modulation of vasoconstriction by EC-MR. While our understanding of the mechanism by which EC-MR exerts site-specific effects on vascular function is far from complete, there are a variety of epigenetic, biomechanical, and biochemical differences among vascular beds that could account for tissue-specific differences in vessel response as reviewed by Aird [205]. Thus, differences in expression of MR itself, other transcription factors and cofactors that interact with the MR, and components of the contractile signaling pathways for each contractile agonist in distinct EC populations could be explored as contributors to this physiology.

Much work remains to determine the mechanism by which EC-MR contributes to coronary vasoconstriction. The finding that decreased endothelin-induced constriction is associated with increased ET_B receptor mRNA in mouse cardiac ECs from EC-MR-KO mice may provide some explanation. Activation of endothelial ET_B receptors by endothelin-1 induces vasorelaxation that counters the constrictive response in the SMCs in an NO-dependent manner[206]. Previous work has also shown a role for MR in post-translational inactivation of ET_B in cultured ECs[102] so EC-MR may regulate endothelin-1 signaling in ECs through multiple mechanisms.

The decreased constriction of the coronary arteries to endothelin-1and thromboxane in EC-MR-KO mice has important clinical implications. Thromboxane is released by activated platelets in the coronary arteries during acute myocardial infarction (MI) thereby contributing to vasoconstriction at the site of plaque rupture that exacerbates myocardial necrosis. Thromboxane-mediated vasoconstriction has been implicated in the morbidity multiple vascular diseases[207]. Endothelin-1 levels are also associated with decreased coronary flow reserve and decreased ejection fraction following MI[97]. In patients presenting with acute MI, studies show a positive correlation between circulating thromboxane A2 or endothelin-1 levels with worse outcomes, including increased risk of poor myocardial perfusion following percutaneous coronary intervention[98;208] . MR antagonists, in contrast, improve clinical outcomes in cardiovascular disease[48;84]. Data from this study demonstrating that EC-MR deletion attenuates thromboxane and endothelin-1-mediated coronary vasoconstriction supports a novel explanation for the benefits of MR antagonism in coronary artery disease in which inhibition of EC-MR decreases coronary vasoconstriction.

There are several limitations to be noted in the interpretation of this study. First, the lack of a specific mouse MR antibody restricts our ability to show loss of MR protein from ECs. To confirm EC-MR deletion, we showed recombination of MR DNA and loss of MR mRNA in ECs from EC-MR-KO mice. We demonstrated a 75% reduction in MR mRNA in cultured EC-MR-KO ECs compared with MR-intact ECs, consistent with reductions in mRNA seen in other endothelial-targeted MR KO mice[65] and in other KO mice made using the VE-Cadherin promoter[209]. Whether this is due to small numbers of SMC in the EC cultures or from incomplete MR deletion from ECs in the mouse model is difficult to discern but incomplete recombination would only underestimate the role of EC-MR in vascular function. Since we identified a vascular-bed specific role for EC-MR in the mesentery and coronary microcirculation, another important limitation is that the results cannot be generalized to other vascular beds. Additional studies are needed to understand the role of EC-MR in regulating blood flow to other critical tissues, by examining cerebral, carotid, renal and skeletal muscle microvessels in this model. Finally, for practical reasons, all experiments were performed only in male mice. There is substantial evidence that male and female mice and humans differ in their vascular function and BP responses[210;211] and MR transcriptional activity was recently found to be modulated by estrogen[212]. Thus, additional studies beyond the scope of this thesis should characterize the role of EC-MR in female mice.

Future directions

This study clarifies that EC-MR does not participate in blood pressure regulation either at baseline or in the setting of hypertension, an important finding because of prior conflicting results[63;65]. It also demonstrates for the first time that EC-MR has vascular bed-specific roles in vasomotor function, a finding that explains the many previous conflicting studies showing variable roles for EC-MR in vasoreactivity[63-65]. However much about EC-MR in vascular function remains to be explored. These studies were all performed in male mice and thus should be repeated in female mice, as EC-MR may play a very different role in that physiology. Likewise, we investigated the role of EC-MR in a limited number of disease models. The most profound clinical benefit for MR antagonism has been found in severe cardiovascular disease including following myocardial infarction and heart failure. Thus use of additional, more severe models may reveal further roles for EC-MR. Finally the mechanism underlying the differences seen in EC-MR-KO mice remains to be elucidated and would be a necessary step in translating these findings to the development of endothelial cell-specific therapies.

Role of EC-MR in atherosclerosis

Chapter 4 described preliminary experiments seeking to clarify the role of EC-MR in the development of atherosclerosis. These studies have shown (1) MR activation by aldosterone exacerbates leukocyte infiltration into the aorta in the setting of high fat diet and hyperlipidemia; (2) pressure myography suggests female EC-MR-KO/ApoE-/- mice are protected from HFD-induced endothelial dysfunction seen in MR-intact/ApoE-/- female mice following HFD, while wire myography studies reveal no difference in female EC-MR-KO/ApoE-/- mice compared to MR-intact/ApoE-/- littermates in endothelial-dependent relaxation after HFD; (3) preliminary experiments show a trend toward male mice lacking EC-MR having decreased atherosclerotic plaque size, but this trend is not observed in female mice.

Future directions

First it was shown that MR activation by aldosterone exacerbates leukocyte infiltration into the aorta in the setting of high fat diet and hyperlipidemia. However, the finding that aldosterone enhances vascular leukocyte infiltration only in the presence of hyperlipidemia does not clarify which cell types require MR

activation to cause this effect. Both endothelial cells and immune cells are potential candidates in mediating this effect and additional experiments would be necessary to determine which cell types are involved. Secondly the mechanism by which hyperlipidemia enables MR activation to enhance infiltration is unclear and requires further investigation.

The assessment of endothelial-dependent vasorelaxation in female MR-intact/ApoE-/- and EC-MR-KO/ApoE-/- mice on HFD yielded in conflicting results. While pressure myography implicated EC-MR in the development of hyperlipidemia-induced endothelial dysfunction, wire myography showed no difference between EC-MR-KO and MR-intact littermates. Several explanations could be responsible for this discrepancy. First, experiments only included three mice per group and thus additional numbers may reveal the difference identified via pressure myography is not reproducible. Alternatively, there could be a role for physiologic intraluminal pressure in modulation of endothelial dysfunction. While pressure myography permits assessment of vessel constriction and relaxation under physiologic pressure, wire myography lacks that component. One study in the literature suggests that endothelial dysfunction exacerbates the myogenic response of increased vasoconstriction with increasing intraluminal pressure[213]. Whether the opposite is true, that intraluminal pressure affects endothelial-dependent relaxation, has not yet been tested but if true and if mediated by EC-MR could account for the difference observed by pressure myography. If the pressure myography finding of a role for EC-MR in hyperlipidemia-induced endothelial dysfunction is reproduced, future experiments can investigate the mechanism by which EC-MR contributes to this effect and whether this effect holds true in male mice.

An in vivo study of male and female EC-MR-KO/ApoE-/- and MR-intact/ApoE-/- littermates assessed atherosclerotic plaque development and lipid content following four-week high fat diet treatment. Preliminary findings (due to very low numbers of mice) suggest a trend toward decreased plaque size in male EC-MR-KO mice compared to MR-intact/ApoE-/- mice that is not observed in females. The key caveat of this study is that no conclusions can be drawn based on such small numbers. However, if the addition of more mice to each group shows this trend to be significant, this experiment would implicate EC-MR in the early development of atherosclerosis, an important finding that could give rise to further experiments to determine the mechanism by which this occurs. Moreover if the trend toward a role for EC-

MR in males but not in females becomes significant, this result would reveal EC-MR to contribute to the sex differences seen in atherosclerosis in animal models and in people. If the finding that EC-MR plays a role in male atherosclerotic development but not in female atherosclerotic development is significant, one explanation could be the ability of estrogen-activated ERα to inhibit MR-mediated transcription[212]. It has previously been shown that EC-MR activates transcription of pro-inflammatory adhesion molecule ICAM-1[13] which is involved in early atherosclerotic plaque development. Thus in females increased estrogen levels could result in increased ERα-mediated inhibition of MR-activated adhesion molecule expression, attenuating movement of immune cells into the plaque and resulting in a decreased role for EC-MR. However in males the loss of EC-MR could result in a substantially decreased influx of immune cells into the plaque and decrease overall plaque size. Addition of more mice to this study will reveal whether these trends are significant.

Summary

Prior to this work, ample literature suggested a role for MR in modulating endothelial function and contributing to cardiovascular disease, but the mechanisms were not clear. In addition, no studies had clearly demonstrated involvement of EC-MR in the cardioprotective effect of estrogen and ERα. While multiple mechanisms undoubtedly contribute to the cardiovascular protection experienced by premenopausal women compared to age-matched men, this work shows for the first time that the inhibition of EC-MR-mediated transcriptional activity by E2 and ERα downregulates mRNA and protein of ICAM-1, an adhesion molecule known to contribute to vascular inflammation. These findings open the door for further investigation into the mechanism by which E2 and ERα modulate MR function in vivo and may lead to identification of downstream targets for cardiovascular therapeutic development.

The field of endothelial MR research also suggested a role for EC-MR in modulation of blood pressure and vasomotor function. Nevertheless previous studies were inconclusive due to the use of EC-MR overexpression of unclear physiologic relevance or EC-MR deletion models in which MR was also lost from leukocyte populations. The work detailed here examines for the first time the role of EC-MR using a truly endothelial-specific MR knockout mouse. Using this tool we confirmed that EC-MR does not contribute to blood pressure regulation at baseline or in response to various pressor stimuli. However, this

same mouse model shows that EC-MR does participate in regulation of vasoreactivity. Thus the use of this mouse model has revealed exciting new roles for EC-MR in the differential regulation of vasomotor function according to vascular bed, in the absence of blood pressure regulation. This work advances the field of EC-MR research by establishing new roles for EC-MR that may be critical in understanding endothelial function in the setting of cardiovascular disease.

It has long been known that MR activation by aldosterone exacerbates atherosclerosis, an inflammatory disease of the vasculature that gives rise to AMI and stroke, two leading causes of death. Our lab has shown that aldosterone enhances vascular infiltration of $CD4^+$ T cells, a critical leukocyte involved in the pathogenesis of atherosclerosis, only in the presence of cardiovascular risk factors including high fat diet and hyperlipidemia. A novel animal model, the EC-MR-KO/ApoE-/-, was used to examine the role of EC-MR in the development of atherosclerosis. Preliminary findings suggest EC-MR may be a key target of MR activation in the setting of hyperlipidemia, with a trend toward decreased plaque size in EC-MR-KO/ApOE-/- male mice compared to their MR-intact/ApoE-/- counterparts. While not yet definitive, this study presents an exciting possibility of a role for EC-MR in atherosclerosis and to a sex specific mechanism that could ultimately yield new therapeutic opportunities to prevent cardiovascular disease.

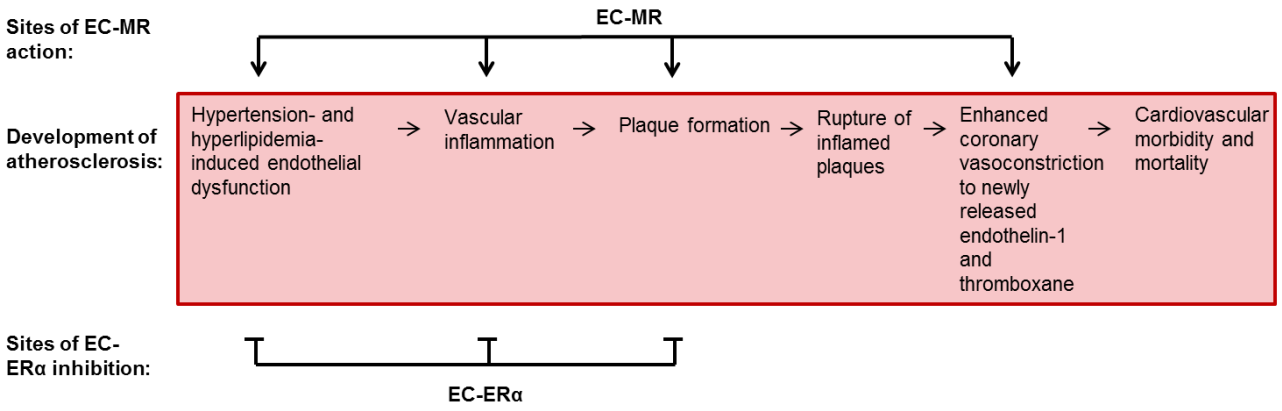

Figure 5.1. Roles for EC-MR in the development of atherosclerosis. This work and others have shown EC-MR to participate in various steps in atherosclerosis development including development of endothelial dysfunction, vascular inflammation, plaque formation, and enhanced vasoconstriction following plaque rupture.

This work demonstrates above all the complexity of the involvement of endothelial MR in cardiovascular physiology and disease. These studies emphasize how in different physiologic or pathologic conditions EC-MR can exert highly variable effects on vessel function and the importance of controlling for the many elements giving rise to vessel health. Future studies seeking to understand the role of endothelial components in cardiovascular disease must account for factors including vessel bed, sex, and overall cardiovascular health before drawing conclusions about endothelial cell function.

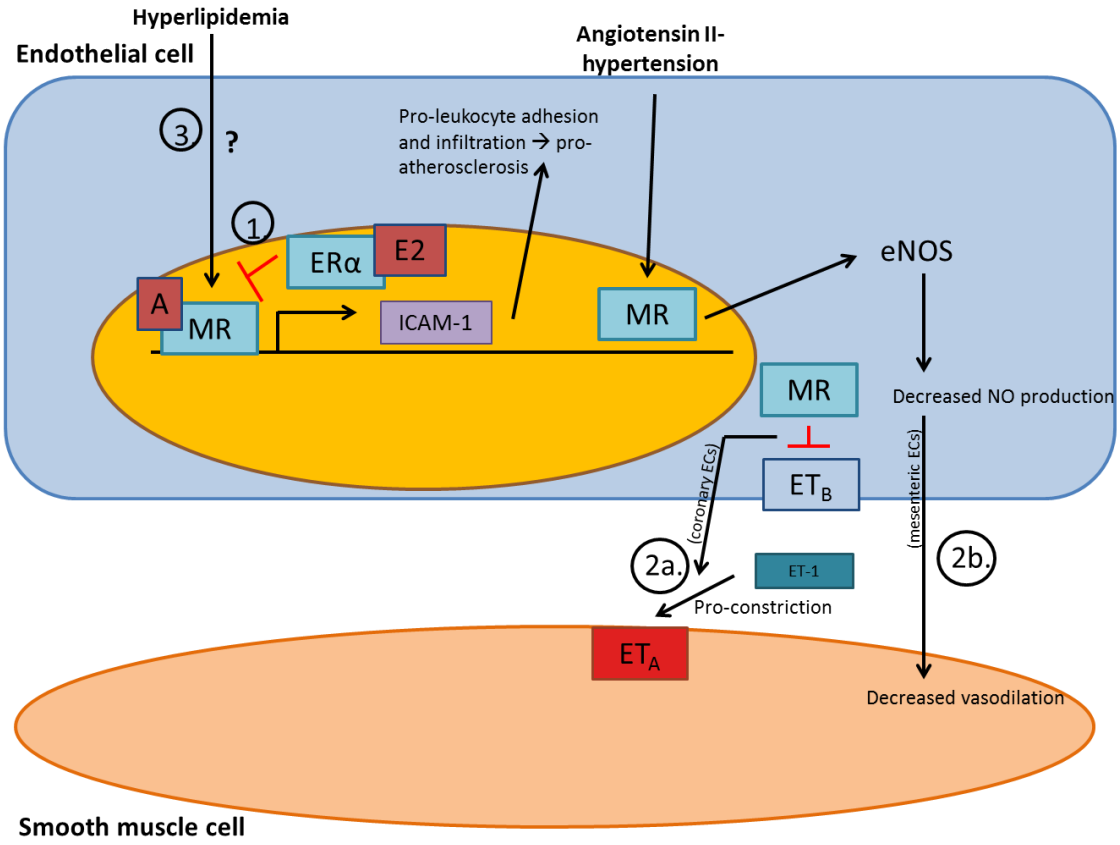

Figure 5.2. Novel roles of endothelial cell mineralocorticoid receptor in vascular function and disease. (1) aldo-stimulated, MR-mediated transcriptional upregulation of ICAM-1 and leukocyte adhesion is attenuated by estrogen-stimulated ERα. (2a) coronary EC-MR enhances vasoconstriction to endothelin-1 and thromboxane agonist. (2b) mesenteric EC-MR attenuates vasodilation in the setting of angiotensin II-stimulated hypertension in an NO-dependent manner. (3) preliminary data suggest EC-MR may contribute to hyperlipidemia-induced increase in leukocyte infiltration that could enhance atherosclerosis development.

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