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## Mineralocorticoid and Estrogen Receptors in Endothelial Cells Coordinately Regulate Microvascular Function in Obese Female Mice

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### Abstract

Obesity impairs endothelial-mediated vasodilation, the earliest step in vascular disease and a contributor to hypertension. We previously demonstrated that endothelial cell mineralocorticoid receptor (MR) deletion prevents obesity-induced microvascular dysfunction in females by increasing nitric oxide-mediated vasodilation. Estrogen receptor alpha (ER $\alpha$ ) can oppose MR function, therefore we hypothesized that ER $\alpha$  mediates the benefits of endothelial MR deficiency.

Females lacking endothelial MR or wildtype littermates were fed control or high fat diet for 20 weeks to cause obesity. MR deletion improved mesenteric artery endothelial-dependent vasodilation in obese females and ER $\alpha$  inhibition *ex vivo* negated this protective effect. Endothelial MR deletion results in significantly more ER $\alpha$  mRNA and protein. *In vitro*, estrogen increases endothelial nitric oxide synthase phosphorylation, which is inhibited by aldosterone and dependent on MR. Both proteins co-immunoprecipitate with striatin and a mimetic peptide that disrupts ER $\alpha$ -striatin binding also decreased MR-striatin interaction. Finally, removing endothelial MR in obese females restored endothelial function by increasing the nitric oxide component of vasodilation. Combined deletion of endothelial ER $\alpha$  negated the benefit of endothelial MR deletion.

These results indicate that endothelial ER $\alpha$  prevents the detrimental effects of MR in obesity by increasing nitric oxide to rescue vasodilation in females. MR and ER $\alpha$  may compete for striatin binding within endothelial cells to regulate nitric oxide. These data identify a novel mechanism that promotes MR antagonism to prevent obesity-induced microvascular dysfunction in females.

### Keywords

mineralocorticoid receptor; estrogen receptor alpha; obesity; endothelial cell; vasodilation; striatin

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## INTRODUCTION

Obesity is a potent cardiovascular disease (CVD) risk factor with rapidly increasing prevalence, particularly in females.<sup>1</sup> Obesity and associated metabolic abnormalities negate pre-menopausal protection of women from CVD, thereby substantially impacting women's health.<sup>2-4</sup> Obesity is associated with impaired endothelial-dependent vasodilation in small resistance arteries, an important contributor to the development of hypertension and an early manifestation of CVD.<sup>5-7</sup> This is important because endothelial dysfunction in small arterioles, rather than large conduit arteries, predicts the 5 year risk of a cardiovascular event and both female sex and increased body mass index are associated with worse endothelial function.<sup>8</sup>

Obesity is associated with increased circulating levels of aldosterone likely due to factors released from adipose tissue that promote aldosterone release.<sup>9-13</sup> Aldosterone activates the mineralocorticoid receptor (MR) in the kidney, which contributes to sodium retention and regulates blood pressure. This may contribute to the association between obesity and hypertension. Circulating aldosterone levels are particularly high in obese females<sup>14</sup>, resulting in excess MR activation. The MR is also expressed in endothelial cells (EC) where it contributes to vascular dysfunction in response to cardiovascular risk factors.<sup>15,16</sup>

EC-MR activation increases reactive oxygen species production<sup>17,18</sup> and decreases available nitric oxide (NO)<sup>19</sup> thereby promoting vascular constriction<sup>20</sup>, inflammation<sup>21,22</sup> and stiffness<sup>23</sup>. Females are more affected as they exhibit more EC-MR expression and this sex difference in MR activation is further exacerbated in obesity due to the higher aldosterone levels in females.<sup>24,25,26</sup> We recently demonstrated that EC-specific MR deletion (EC-MR-KO) rescues female mice from obesity-induced endothelial dysfunction, however the mechanism has not been determined.

Like aldosterone, the female sex hormone estrogen is a steroid hormone which binds to intracellular estrogen receptors (ER). Endothelial ER alpha (ER $\alpha$ ) isoform activation accelerates reendothelialization after vascular injury and promotes vasodilation by increasing NO production via endothelial NO synthase (eNOS). ER $\alpha$  binds to the scaffolding protein striatin via amino acids 176-253 and this interaction is necessary for estrogen to activate eNOS by phosphorylation to induce NO production.<sup>27,28</sup> MR also binds to striatin when activated by aldosterone, inducing EC production of reactive oxygen species.<sup>17</sup>

Obese female mice have impaired microvascular endothelial function and we previously demonstrated that EC-MR deletion preserves vasodilation by preserving nitric oxide synthase (NOS) component of vasodilation specifically in females.<sup>26</sup> Here we explore the mechanism by which EC-MR-KO protects female mice from obesity-induced endothelial dysfunction and hypothesize that EC-ER $\alpha$  may mediate these benefits.

## METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. See supplemental material for detailed methods.

## Animal Studies

Experiments were approved by Tufts University Institutional Animal Care and Use Committee and conducted in accordance with the Guide for Care and Use of Laboratory Animals. Female mice were euthanized under isoflurane. Small mesenteric arteries were mounted in a wire myograph, precontracted with phenylephrine (PE) and endothelial-dependent-dilation to acetylcholine was measured.

## Molecular Biology

EA.hy926 cells (human endothelial cell hybrid line) stably expressing ER $\alpha$  or HEK293 cells were cultured and treated as previously described<sup>27,29</sup>

## Statistics

Data were collected and analyzed by genotype and/or treatment blinded investigators. Statistical analysis was performed in GraphPadPrism version 8. Four groups and concentration response curves were analyzed by 2-way ANOVA with Bonferroni post hoc. Three groups were analyzed by one-way ANOVA with Sidak post hoc. Data is presented as mean  $\pm$  standard error. Significance was set at  $p < 0.05$ .

## RESULTS

### Female Mice Lacking the Mineralocorticoid Receptor in Endothelial Cells are Protected from Obesity-Induced Endothelial Dysfunction

Female EC-MR-WT and EC-MR-KO littermates were randomized to normal chow or high fat diet for 20 weeks. High fat feeding resulted in a significant increase in body weight, fasting glucose, and serum aldosterone when compared to lean littermates consistent with the development of obesity with metabolic abnormalities (Table S1). There was no significant difference in these parameters between EC-MR-WT and EC-MR-KO mice. Endothelial-dependent vasodilation to ACh was quantified *ex vivo* in 2<sup>nd</sup>-3<sup>rd</sup> order mesenteric arteries. Consistent with our prior findings<sup>26</sup>, EC-MR deletion did not impact endothelial-dependent vasodilation in lean female mice as evidenced by near complete peak vasodilation (Figure 1A). In obese female mice (Figure 1B), 86% peak dilation occurs in EC-MR-WT mice while obese EC-MR-KO females have significantly improved endothelial function with preserved vasodilation capacity.

### Estrogen Receptor Alpha (ER $\alpha$ ) Contributes to the Vascular Benefits of EC-MR Deletion in Obese Females

Since the benefit of EC-MR deletion is specific to females<sup>26</sup>, we next tested whether ER might contribute to the improved vasodilation in obese EC-MR-KO females. Mesenteric arteries from obese female mice were incubated *ex vivo* for 30 minutes with ICI181780 (ICI), an estrogen receptor antagonist (inhibits ER $\alpha$  and ER $\beta$ ). ICI inhibition in obese females abrogated improved dilation seen in EC-MR-KO (Figure 1B). Hence, there was no longer any genotype difference in endothelial function with ICI treatment (Figure 2A). To determine which ER isoform mediates this effect, arteries were incubated with MPP, a specific ER $\alpha$  inhibitor (Figure 2B), or PHTPP, a specific ER $\beta$  inhibitor (Figure 2C). ER $\alpha$

inhibition did not impact vasodilation in EC-MR-WT obese females but significantly attenuated ACh dilation in EC-MR-KO obese female arteries (Figure 2B) supporting that ER $\alpha$  contributes to the improved endothelial function in obese mice lacking EC-MR. There was no significant effect of acute ER $\beta$  inhibition on vasodilation in obese females with or without EC-MR (Figure 2C). In lean females, neither ER $\alpha$  nor ER $\beta$  inhibition impacted ACh dilation (Figure S1). We next examined ER $\alpha$  expression in mesenteric arteries from EC-MR-WT and EC-MR-KO mice. ER $\alpha$  mRNA and protein was significantly increased in both lean and obese female mice lacking EC-MR (Figure 2D). ER $\alpha$  protein was also significantly increased in obese versus lean mice of the same genotype (Figure 2E). In human EC, MR inhibition with spironolactone also increased endogenous levels of ER $\alpha$  protein (Figure S2). Together, these data support that ER $\alpha$  expression is increased in vessels from mice lacking EC-MR and that ER $\alpha$  is contributing to the benefits of EC-MR deletion.

### **Aldosterone Inhibits Estrogen-Induced eNOS Phosphorylation via MR in Human Endothelial Cells**

EC ER $\alpha$  activation by estrogen increases eNOS activity by phosphorylation of serine 1177.<sup>27</sup> As females are exposed to elevated aldosterone when they become obese, we examined the impact of aldosterone on estrogen-induced eNOS phosphorylation. ER $\alpha$ -expressing human EAhy926 ECs were treated with MR siRNA or scrambled siRNA control and MR knock down was confirmed (Figure 3A). Next, these cells were treated with physiologically relevant concentrations of aldosterone, estrogen or both hormones with appropriate vehicle controls for 20 minutes. In control siRNA-treated ECs, aldosterone alone had no effect on eNOS phosphorylation and, as expected, estrogen significantly increased eNOS phosphorylation at S1177. When MR is present, addition of aldosterone significantly attenuated the estrogen-induced increase in eNOS phosphorylation (Figure 3B), showing that aldosterone inhibits estrogen rapid signaling in EC in vitro. In MR siRNA-treated cells, estrogen still increased eNOS phosphorylation. However, when MR was knocked down, aldosterone had no impact on estrogen-induced on eNOS phosphorylation (Figure 3C). These data show that aldosterone prevents estrogen induction of eNOS activity in ECs in a MR-dependent manner.

### **MR and ER Interact with Striatin in Endothelial Cells and Aldosterone Decreases the ER $\alpha$ -Striatin Interaction**

ER $\alpha$  must interact with the scaffolding protein striatin in EC caveolae to regulate eNOS phosphorylation.<sup>27</sup> MR is also known to regulate signaling via interactions with striatin.<sup>17</sup> (see Figure 4A for model). Thus, we tested the impact of each hormone on the relative amount of MR or ER $\alpha$  bound to striatin by co-immunoprecipitation. ECs were treated with aldosterone +/- estrogen as in Figure 3 and cell lysates were immunoprecipitated with anti-striatin antibody and immunoblotted for striatin, MR and ER $\alpha$  (Figure 4B). The amount of MR or ER $\alpha$  pulled down with striatin was quantified and normalized to the total amount of striatin pulled down and expressed as a fold change relative to vehicle treatment (Figure 4C). Aldosterone significantly increased the amount of MR bound to striatin and this was prevented by estrogen. Estrogen significantly increased the amount of ER $\alpha$  bound to striatin and, as with eNOS phosphorylation in Figure 3B, estrogen induction of ER $\alpha$ -striatin complex formation was prevented by aldosterone.

Previous work characterized the ER $\alpha$  binding domain that interacts with striatin and found that overexpression of the ER $\alpha$  peptide containing this region (amino acids 176-253, Figure 4D) prevents ER $\alpha$  binding to striatin and inhibits ER $\alpha$  signaling to induce eNOS phosphorylation.<sup>27</sup> Thus, we examined whether overexpression of the ER $\alpha$  amino acid 176-253 would modulate ER or MR binding to striatin when the receptors are co-expressed. HEK cells were transfected with plasmids to express both MR and ER $\alpha$  with or without the flag tagged ER176-253 peptide. Cell lysates were immunoprecipitated with striatin and blotted for MR or ER $\alpha$  (Figure 4E). As expected, expression of the peptide prevented estrogen induced recruitment of ER $\alpha$  to striatin. The same ER $\alpha$  176-253 peptide also significantly decreased basal MR binding to striatin and prevented further aldosterone-induced MR recruitment to striatin (Figure 4F).

### **EC-ER $\alpha$ Deletion Eliminates the Protective Impact of EC-MR-KO on Microvascular NO Contribution to Vasodilation in Obese Females**

The data presented thus far are consistent with a model in which when MR is deleted from ECs in obese females, the combination of increased ER $\alpha$  expression and enhanced recruitment of ER $\alpha$  to striatin results in the increased eNOS activity to enhance vasodilation in obese EC-MR-KO mice. To test this model, a double knockout mouse was created lacking MR and ER $\alpha$  (EC-ER $\alpha$ /MR-KO) specifically in EC. Lung tissue rich in ECs was used to confirm recombination of MR and ER $\alpha$  via PCR (Figure 5A). EC-MR-KO and EC-ER $\alpha$ /MR-KO female mice and their respective WT littermates were fed a high fat diet for 20 weeks resulting in obesity with metabolic dysfunction with no difference in weight, glucose or aldosterone in obese EC-ER $\alpha$ /MR-KO compared to EC-ER $\alpha$ /MR-WT littermates (Table S1). There was also no difference in basal lumen diameter or phenylephrine constriction in any of the groups (Table S2). Again, EC-MR-KO improved vasodilation relative to WT but added deletion of ER $\alpha$  mitigated the benefits of EC-MR deletion and further impaired endothelial function in lean (Figure S3) as well as obese EC-ER $\alpha$ /MR-KO mice relative to WT or EC-MR-KO littermates (Figure 5B).

To determine the contribution of NOS to vasodilation in these mice, ACh concentration-response curves were compared between arteries with or without the NOS inhibitor LNAME. Figure 5C shows the vasodilation curves for all of the WT control animals revealing that the obese WT females regardless of the presence of floxed alleles (EC-MR-WT and EC-ER $\alpha$ /MR-WT) have overlapping vasodilation curves with a similar decline in vasodilator response to NOS inhibition (Figure 5C). Next, NOS inhibition in obese EC-MR-KO and the EC-ER $\alpha$ /MR double KO animals was compared (Figure 5D). As we previously demonstrated, obese female EC-MR-KO mice have preserved vasodilation and this is due to an increase in the NOS component of vasodilation (Figure 5D, black curves). EC-ER $\alpha$ /MR-KO arteries had impaired vasodilation that was further decreased by NOS inhibition. The difference in area between the curves  $\pm$  LNAME was calculated to determine the NOS component of vasodilation (Figure 5E). WT mice (EC-MR-WT and EC-ER $\alpha$ /MR-WT) were combined as their curves were nearly identical in Figure 5C. The significant increase in NOS-mediated vasodilation in EC-MR-KO vs EC-MR-WT obese female mice is lost in EC-ER $\alpha$ /MR-KO making the NOS component significantly lower in double KO compared to EC-MR-KO obese female mice (Figure 5E). These data support that EC-ER $\alpha$  is necessary

for the benefits of MR deletion on female microvascular function and this benefit is mediated by an increase in the NOS component of vasodilation.

## DISCUSSION

This is the first study to examine the interconnected signaling of EC-MR and EC-ER $\alpha$  in the microvasculature of obese female mice. EC-specific deletion of MR protects from obesity-induced vascular dysfunction and this study identified the mechanisms by showing that; 1) The improvement in *ex vivo* microvascular dilation upon EC-MR deletion is mediated functionally by ER $\alpha$ , 2) Microvascular ER $\alpha$  mRNA and protein expression is upregulated when EC-MR is deleted or inhibited, 3) In human ECs, MR activation prevents estrogen-induced eNOS phosphorylation, 4) ER $\alpha$  and MR both bind to striatin, inhibit each other's binding when activated by hormone, and can be displaced by the ER $\alpha$  striatin-binding peptide, and 5) In vivo, using our novel EC-ER $\alpha$ /MR double KO mouse, EC-ER $\alpha$  is necessary for the improvement in the NOS component of vasodilation in obese females lacking EC-MR. The results are consistent with the model in Figure 6 in which enhanced EC-MR activation in obese females; 1) suppresses ER $\alpha$  expression, 2) decreases ER $\alpha$  recruitment to the caveolae by striatin, and 3) impairs NOS contribution to vasodilation. This results in obesity-induced endothelial dysfunction (Figure 6, left), an important contributor to adverse cardiovascular outcomes in obese women. Conversely, when we remove/inhibit EC-MR *in vitro* or *in vivo* (Figure 6, right), microvascular ER $\alpha$  expression is increased and aldosterone no longer prevents estrogen-induced eNOS phosphorylation (Figure 3) resulting in increased microvascular NOS vasodilatory contribution and improved endothelial function (Figure 5E). We conclude that the balance between ER $\alpha$  and MR signaling maintains vascular homeostasis in female ECs, but when MR activation predominates in obesity, the benefit of estrogen on female vasodilation is lost. This is clinically important as obesity is associated with elevated aldosterone and impaired endothelial function in animals and humans, exacerbated in females, and results in detrimental cardiovascular outcomes in obese woman. The new data support future clinical testing of MR inhibition to restore endothelial function specifically in obese women.

This study sheds light on a controversy regarding the impact of aldosterone on EC function. Animal and human studies suggest that aldosterone impacts endothelial function although there are conflicting studies showing improved or worsening of vasodilation depending on the model or the patient population tested (reviewed in <sup>30</sup>). Similarly, *in vitro* studies vary. Some show aldosterone increases, decreases, or has no effect on eNOS phosphorylation or NO production.<sup>17,19,31,32</sup> The current study suggests that the impact of rapid aldosterone signaling via EC-MR on eNOS may depend on the presence of ER $\alpha$  or estrogen. For example, in Figure 3B, aldosterone alone has no impact on eNOS phosphorylation in cells grown in estrogen-free media. However, when estrogen is present, comparison of vehicle to aldosterone shows a decrease in eNOS phosphorylation, suggesting that MR is blocking eNOS activation in ECs. Since ER activators are present in phenol red-containing culture medium and in serum, different studies in the literature are effectively testing the impact of aldosterone under different conditions. In addition, ER expression in ECs changes with culture conditions and generally declines with cell passage. Some of the discrepancies in the

literature regarding aldosterone and eNOS phosphorylation may be due to differences in ER $\alpha$  or estrogen that are not intentional.<sup>31,32</sup>

There are several potential mechanisms by which ER and MR signaling interact in ECs. ER $\alpha$  mRNA and protein expression in mesenteric arteries increased when EC-MR was deleted and ER $\alpha$  protein increased when EC-MR was inhibited *in vitro*. This supports a mechanism in which MR suppresses ER $\alpha$  mRNA transcription or stability in ECs resulting in increased ER $\alpha$  expression when MR is deleted or inhibited. A prior study in Kupffer cells similarly showed that MR suppresses ER $\alpha$  expression as myeloid-MR-KO increased ER $\alpha$  and MR overexpression repressed ER $\alpha$  expression.<sup>33</sup> MR and ER are also both hormone-activated transcription factors and prior studies reveal that ER $\alpha$  blocks MR regulation of inflammatory gene transcription in EC which may contribute to sex differences in atherosclerosis.<sup>21,34</sup> MR and ER both bind to striatin where they initiate rapid signaling cascades that regulate EC function. This study shows that MR activation decreases ER $\alpha$  recruitment to striatin and estrogen activation of eNOS. The finding that the peptide of ER $\alpha$  that dissociates ER $\alpha$  from striatin also dissociates MR from striatin suggests that the two receptors may compete for binding to the same site on striatin or form a tri-protein complex that requires a similar domain. Further studies are needed to clarify the details of these molecular interactions but overall, ER $\alpha$  and MR interact in at least three ways in ECs to integrate hormonal signals into changes in EC function.

The interaction between ER $\alpha$  and MR signaling in ECs has important clinical implications for our understanding of the impact of obesity on women's cardiovascular health. Although premenopausal women are protected from cardiovascular diseases including hypertension, myocardial infarction, and stroke compared to age-matched men, this protection is lost in women with obesity and metabolic syndrome resulting in rising CVD risk in middle aged women.<sup>2-4</sup> High body mass index is directly correlated with plasma aldosterone concentration in hypertensive humans<sup>35</sup>, even those treated with antihypertensives.<sup>36</sup> Obese women exhibit higher circulating aldosterone, more hypertension, and a greater increase in cardiovascular related morbidity and mortality than obese men.<sup>1-3,14,37</sup> The first cardiovascular consequence of excess adiposity is microvascular endothelial dysfunction.<sup>38</sup> Our data suggests that in younger obese females, excess aldosterone activating EC-MR may negate the protection typically afforded by the presence of circulating estradiol and activation of endothelial ER $\alpha$ . Translating this into human therapeutic intervention are important next steps to combat the increase in cardiovascular-related mortality of premenopausal females. Small clinical trials (25 women in two studies) have specifically shown a greater benefit of MR antagonism in obese women.<sup>39,40</sup> Obese women have a more beneficial blood pressure drop to MR antagonism than men.<sup>40</sup> Animal studies also support the notion that MR antagonism could be particularly efficacious in obese females<sup>21,23,24,26,41-43</sup> to prevent vascular dysfunction, but there is still a need for larger clinical studies in humans. MR antagonist are typically a fourth line anti-hypertensive, but this study and others provide a rationale for earlier use of MR antagonism to maintain endothelial function in obese women.

Some limitations to this study should be acknowledged. Testing acute ER inhibition on vasodilation using MPP or ICI182170 used whole arteries treated with inhibitors, thereby

blocking ER $\alpha$  in smooth muscle as well as the EC. Thus, we cannot conclude that the result is mediated only by blocking EC-ER. However, in our EC specific KO mouse model, ER $\alpha$  combined with MR deletion recapitulated the inhibitor studies and further impaired EC-dependent vasodilation. The more profound response in the tissue-specific model could be due to differences between ER function in EC versus smooth muscle as well as the combined loss of acute signaling and longer term gene expression regulation by ER $\alpha$  *in vivo* that are important in maintenance of vascular physiology.<sup>44</sup> This is consistent with the finding that lean EC-MR/ER $\alpha$ -KO mice have impaired endothelial function while acute ER $\alpha$  inhibition has no effect. We specifically focused on female (not male) microvascular function since our prior study showed that EC-MR deletion had microvascular benefits only in obese female mice. Future studies will be needed to assess whether these MR/ER interactions are relevant in males. Whether these mechanisms contribute to other important resistance artery functions including blood pressure regulation or vascular remodeling remains to be explored. Despite these limitations, this study describes a novel mechanism for vascular dysfunction in premenopausal obese females, provides insight into sex differences in the impact of metabolic risk factors on CVD, and supports testing of MR inhibition as a sex-specific therapeutic strategy to mitigate the adverse cardiovascular complications of obesity in the rapidly growing population of obese women with metabolic syndrome.

### Perspectives

The incidence of obesity is rapidly rising, particularly in women, and the associated metabolic abnormalities mitigate the protection afforded to premenopausal woman from cardiovascular disease. This study provides preclinical data supporting a novel molecular mechanism for this observation. Obesity is associated with elevated serum aldosterone and increased expression of the aldosterone-binding mineralocorticoid receptor (MR) in endothelial cells (EC). This study demonstrates that obesity-induced microvascular endothelial dysfunction in females depends on EC-MR. When EC-MR is deleted, endothelial function is improved due to an increase in the nitric oxide synthase (eNOS) component of vasodilation. Enhanced microvascular endothelial function in females is mediated by estrogen receptor alpha (ER $\alpha$ ) in ECs and this is impaired by EC-MR activation in obesity through two potential mechanisms: 1) the presence of EC-MR suppresses ER $\alpha$  expression in arteries; and 2) EC-MR activation decreases estrogen-induced activation of eNOS by preventing ER $\alpha$ -striatin interaction. These mechanisms may explain the disproportional impact of obesity on vascular function in premenopausal obese females and supports testing of MR inhibition as a sex-specific therapeutic strategy to mitigate the adverse cardiovascular complications of obesity in women.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations:

<b>ACh</b>	acetylcholine
<b>Aldo</b>	aldosterone
<b>CVD</b>	cardiovascular disease
<b>E2</b>	estradiol
<b>EC</b>	endothelial cell
<b>eNOS</b>	endothelial nitric oxide synthase
<b>ER</b>	estrogen receptor
<b>ER<math>\alpha</math></b>	estrogen receptor alpha
<b>ER<math>\beta</math></b>	estrogen receptor beta
<b>KO</b>	knockout
<b>MPP</b>	1,3- <i>Bis</i> (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1 <i>H</i> -pyrazole dihydrochloride
<b>MR</b>	mineralocorticoid receptor
<b>NOS</b>	nitric oxide synthase
<b>PE</b>	phenylephrine
<b>PHTPP</b>	4-[2-Phenyl-5,7- <i>bis</i> (trifluoromethyl)pyrazolo[1,5- <i>a</i> ]pyrimidin-3-yl]phenol
<b>VE-Cad</b>	Vascular endothelial cadherin
<b>WT</b>	wild type

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### Novelty and Significance

#### What is New:

- Microvascular dysfunction in obese females is mediated by the mineralocorticoid receptor (MR) in endothelial cells (EC) by suppressing the ability of estrogen receptor alpha (ER $\alpha$ ) to enhance nitric oxide production.
- Mechanistically, EC-MR suppresses expression of microvascular ER $\alpha$  and prevents ER $\alpha$  recruitment to caveoli by striatin where it activates endothelial nitric oxide synthase.

#### What is Relevant:

- The prevalence of obesity is increasing, especially in women, resulting in increased activation of the MR which promotes hypertension and cardiovascular disease.
- This study provides an explanation for the loss of cardiovascular protection in premenopausal women with obesity and metabolic syndrome.

**Summary:**

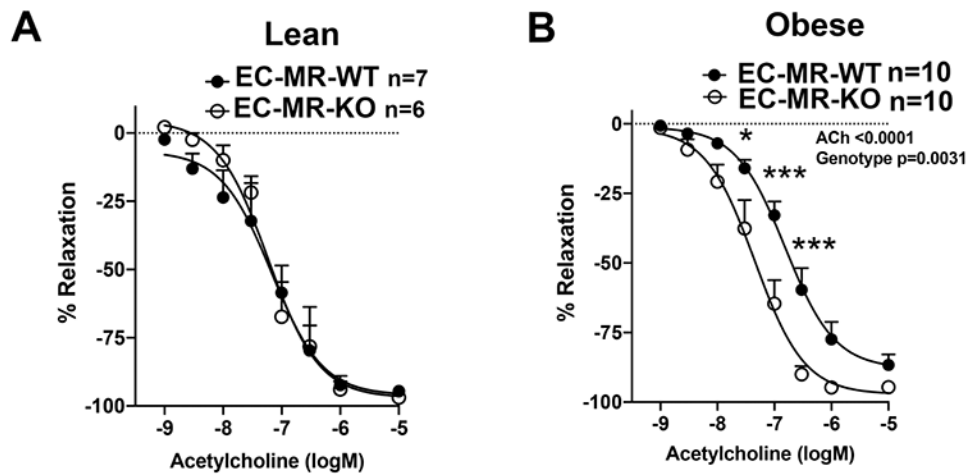
MR in endothelial cells prevents vascular protection mediated by ER $\alpha$  in obese females supporting the potential for MR inhibition to protect obese women from cardiovascular disease.

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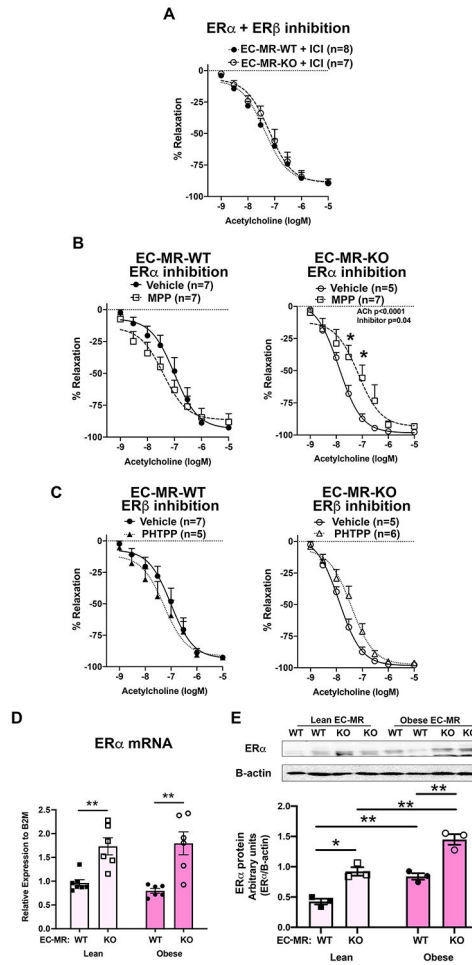
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**Figure 1. Endothelial Mineralocorticoid Receptor Knockout (EC-MR-KO) Improves Obesity-Induced Microvascular Vasodilation Impairment.**

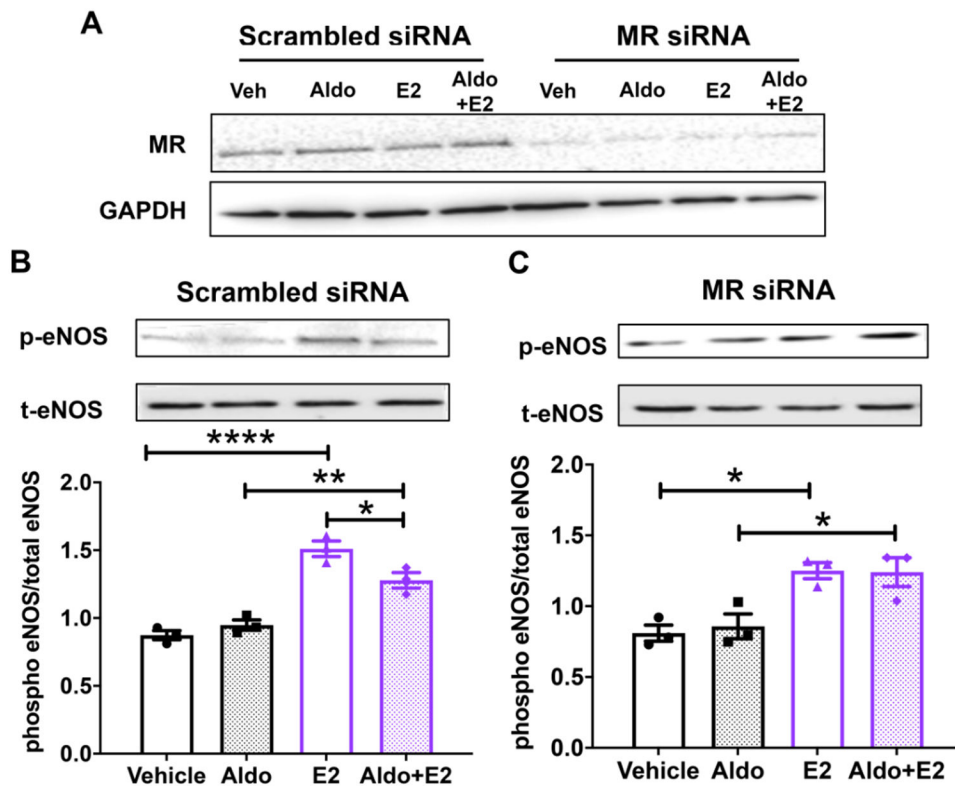
Female EC-MR intact (EC-MR-WT) and EC-MR-KO mice were fed normal (lean) or high fat diet (obese) for 20 weeks and endothelial-dependent vasodilation was measured *ex vivo* using acetylcholine (ACh). **(A)** In lean females, there is no difference in vasodilation comparing EC-MR-WT (closed circles) versus EC-MR-KO (open circles). **(B)** In obese females, vasodilation is improved in EC-MR-KO versus EC-MR-intact. \* $p < 0.05$ , \*\*\* $p < 0.001$ , 2-way repeated measures ANOVA, Bonferroni post hoc.



**Figure 2. Estrogen Receptor Alpha (ERα) Contributes to the Vascular Benefits of Endothelial MR Deletion in Obesity.**

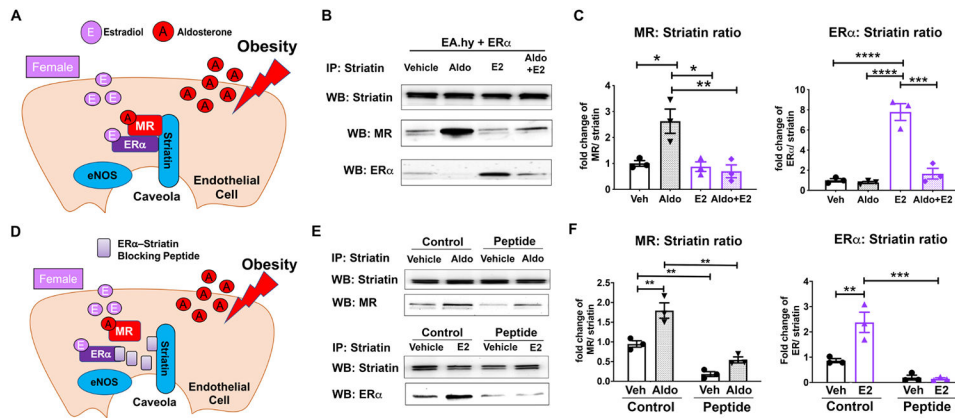
Mesenteric arteries from obese females were incubated with ER inhibitors for 30 minutes and ACh vasodilation measured. **(A)** Incubation with the combined ERα and ERβ inhibitor ICI182,780 eliminated the improved dilation previously seen in arteries from obese EC-MR-KO mice. **(B)** Incubation with vehicle versus the ERα-specific inhibitor MPP in EC-MR-WT arteries and EC-MR-KO arteries. ERα inhibition significantly decreased endothelial-dependent vasodilation in obese EC-MR-KO mice. \*p<0.05, 2-way repeated measures ANOVA, Bonferroni post-hoc test. **(C)** Incubation with vehicle or the ERβ-specific inhibitor PHTPP in obese female EC-MR-WT arteries and obese female EC-MR-KO arteries. ERβ inhibition did not significantly impact vasodilation. 2-way repeated measures ANOVA, Bonferroni post-hoc test. **(D)** ERα mRNA and **(E)** protein expression quantified in mesenteric artery tissue from lean and obese EC-MR-WT and EC-MR-KO females. \*p<0.05, \*\*p<0.01, 2-way ANOVA, Bonferroni post-hoc test.





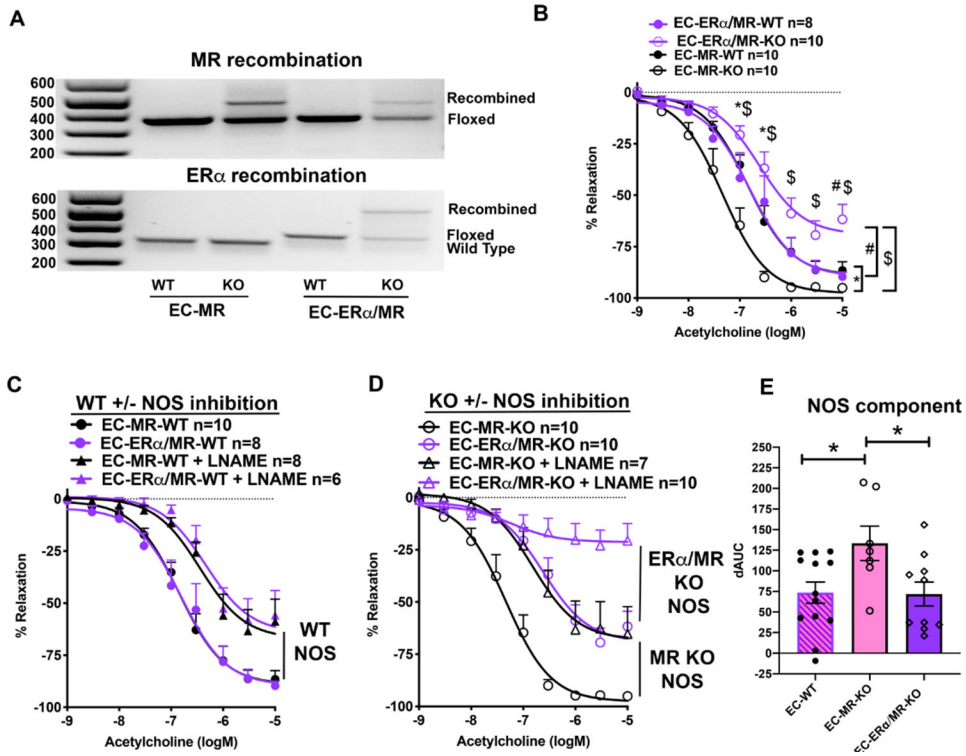
**Figure 3. Aldosterone Inhibits Estrogen-Induced eNOS Phosphorylation via MR in Endothelial Cells**

(A) EAhy endothelial cells stably expressing ER $\alpha$  were treated with scrambled control siRNA or MR siRNA for 48 hours and MR knock down was demonstrated by immunoblotting. (B-C) Cells were treated for 20 minutes with vehicle, 10 nM aldosterone (Aldo), 10 nM estradiol (E2) or E2+Aldo. Ratio of phospho-eNOS to total eNOS was quantified by immunoblotting: (B) in control cells with MR or (C) with MR knock down. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ , N=3, one-way ANOVA with Bonferroni post hoc.



**Figure 4. MR and ER Complex with Striatin in Endothelial Cells and Aldosterone Decreases the ER $\alpha$ -Striatin Interaction**

(A) Schematic showing an endothelial cell (EC) exposed to increased aldosterone during obesity, which binds to MR. In females, estradiol is bound to ER $\alpha$ . Both receptors can bind to striatin in EC membrane caveolae to potentiate rapid signaling. (B) Representative immunoblot of EaHY human EC line with stable ER $\alpha$  expression treated with vehicle, 10 nM aldosterone (Aldo), 10 nM estradiol (E2) or E2+Aldo. Cells lysate was immunoprecipitated with anti-striatin antibody and then immunoblotted for striatin, MR, or ER $\alpha$ . (C) The amount of MR or ER bound to striatin was quantified. N=3-4. (D) Schematic showing a cell expressing an ER $\alpha$  mimetic blocking peptide (amino acids 176-253) which prevents ER $\alpha$ -striatin interaction. (E) Representative blots of HEK293 cells overexpressing MR and ER $\alpha$  +/- co-expression of ER $\alpha$ -striatin blocking peptide. Cells were treated with vehicle, Aldo or E2 for 20 minutes and cell lysates were immunoprecipitated with striatin antibody and immunoblotted for striatin, MR, and ER $\alpha$ . (F) The amount of MR or ER $\alpha$  normalized to striatin was significantly decreased with co-expression of the ER $\alpha$  -striatin disrupting peptide when compared to vector plasmid (Control). N=3. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 via one-way ANOVA with Bonferroni post hoc



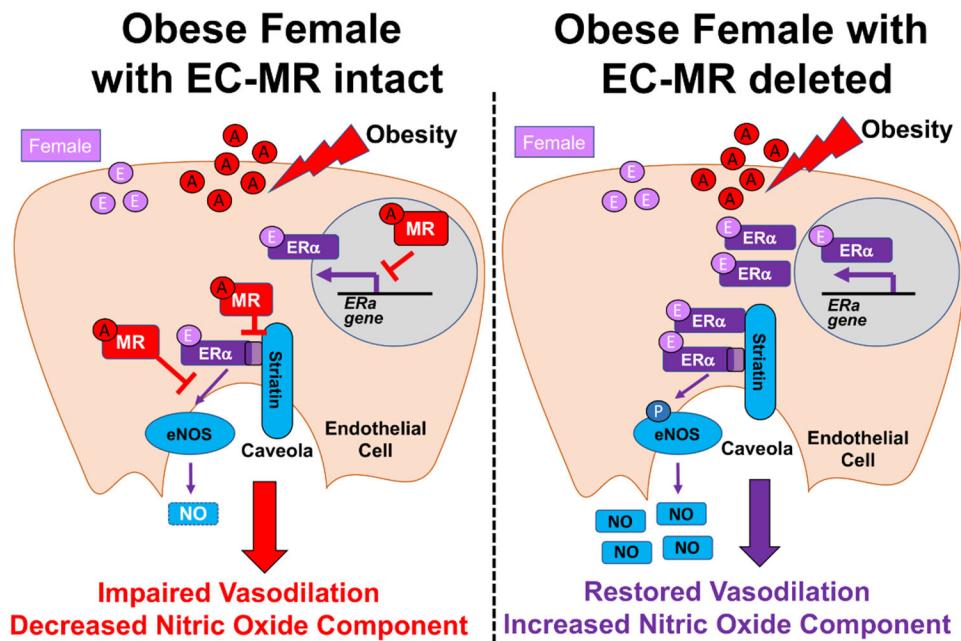
**Figure 5. Combined ERα and MR Deletion in EC Negates Improved EC-MR-KO Endothelial Dependent Vasodilation by decreasing NO Contribution.**

(A) Representative images of PCR confirming gene recombination using DNA from EC-rich lung tissue from EC-MR-WT/KO and EC-ERα/MR-WT/KO mice. PCR indicates the wild type MR allele, floxed MR allele and recombined MR (top image) and wild type ERα, floxed ERα, and recombined ERα (bottom image).

(B) ACh vasodilation is impaired in EC-ERα/MR-KO females. EC-MR-WT and KO curves from Figure 1A are replotted here in black and compared directly. 2-Way repeated measures ANOVA, Bonferroni post hoc. \*p<0.05 EC-MR-WT vs EC-MR-KO; #p<0.05 EC-ERα/MR-WT vs EC-ERα/MR-KO; \$p<0.05 EC-ERα/MR-KO vs EC-MR-KO.

(C) Nitric oxide synthase (NOS) contribution to vasodilation in obese WT females. (D) NOS contribution in obese EC-MR-KO females and EC-ERα/MR-KO females.

(E) NOS contribution to vasodilation was calculated as difference in the area under the curve (dAUC) between ACh dilation and ACh+NOS inhibition in each genotype. WT females were combined from both WT groups (EC-MR-WT and EC-ERα/MR-WT). \*p<0.05, one-way ANOVA with Sidak post-hoc.



**Figure 6. Model for the Interaction of MR and ER $\alpha$  in Endothelial Cells to Regulate Microvascular Function in Obese Females**

Obesity is associated with increased aldosterone (A) in females which activates the MR in endothelial cells (left). MR in ECs decreases ER $\alpha$  mRNA and protein expression in resistance arteries. MR also decreases ER $\alpha$  binding to striatin and impairs estrogen-induced eNOS phosphorylation and the contribution of NOS to microvascular dilation. In EC-MR-KO females (right), aldosterone is increased but cannot bind to MR. Therefore, the microvasculature contains more ER $\alpha$  protein and ER $\alpha$  can interact more readily with striatin. This results in ER $\alpha$ -dependent increase in NOS contribution to vasodilation and prevents EC dysfunction in obese females.