Estrogen Receptor-β in the Paraventricular Nucleus and Rostroventrolateral Medulla Plays an Essential Protective Role in Aldosterone/Salt-Induced Hypertension in Female Rats

Baojian Xue, Zhongming Zhang, Terry G. Beltz, Ralph F. Johnson, Fang Guo, Meredith Hay, Alan Kim Johnson

Abstract—The identification of the specific estrogen receptor (ER) subtypes that are involved in estrogen protection from hypertension and their specific locations in the central nervous system is critical to our understanding and design of effective estrogen replacement therapies in women. Using selective ER agonists and recombinant adeno-associated virus (AAV) carrying small interference (si) RNA to silence either ERα (AAV-siRNA-ERα) or ERβ (AAV-siRNA-ERβ), the present study investigated regional specificity of different ER subtypes in the protective actions of estrogen in aldosterone (Aldo)-induced hypertension. Intracerebroventricular infusions of either diarylpropionitrile, a selective ERβ agonist, or propyl-pyrazole-trioli, a selective ERα agonist, attenuated Aldo/NaCl-induced hypertension in ovariectomized rats. In contrast, intracerebroventricular injections of siRNA-ERα or siRNA-ERβ augmented Aldo-induced hypertension in intact females. Site-specific paraventricular nucleus (PVN) or rostroventrolateral medulla (RVLM) injections of siRNA-ERβ augmented Aldo-induced hypertension. However, rats with PVN or RVLM injections of siRNA-ERα did not significantly increase blood pressure induced by Aldo. Real-time polymerase chain reaction analyses of the PVN and RVLM of siRNA-injected rat confirmed a marked reduction in the expression of ERα and ERβ. In cultured PVN neurons, silencing either ERα or ERβ by culturing PVN neurons with siRNA-ERα or siRNA-ERβ enhanced Aldo-induced reactive oxygen species production. Ganglionic blockade after Aldo infusion showed an increase in sympathetic activity in ERβ knockout rats. These results indicate that both PVN and RVLM ERβ, but not ERα in these nuclei, contribute to the protective effects of estrogen against Aldo-induced hypertension. The brain regions responsible for the protective effects of estrogen interaction with ERα in Aldo-induced hypertension still need to be determined. (Hypertension. 2013;61:1255-1262.) ● Online Data Supplement

Key Words: aldosterone ■ blood pressure ■ central nervous system ■ estrogen receptors

The incidence and severity of hypertension have been shown to be lower in premenopausal women than age-matched men, as well as in female animals of hypertensive models.1-3 Estrogen has been demonstrated to have beneficial effects on cardiovascular function through actions not only on the heart and vasculature but also on the central nervous system (CNS).4 Microinjections of estrogen into the parabrachial nucleus, the nucleus of the solitary tract (NTS), or the rostroventrolateral medulla (RVLM) of male rats have been shown to decrease resting blood pressure (BP) and increase baroreceptor sensitivity.5,6 In contrast, microinjection of estrogen into the hypothalamic paraventricular nucleus (PVN) has no effect on resting BP and heart rate (HR), but attenuates an l-glutamate–induced increase in BP.7 These results suggest that the effects of estrogen on BP and HR in central autonomic nuclei are regionally specific.

It is well known that estrogen actions on cardiovascular hemodynamics are mediated by at least 2 classical estrogen receptor (ER) subtypes, ERα and ERβ, which seem to be genetically and functionally distinct.8 Both ER subtypes are expressed in many autonomic nuclei of the CNS, such as the NTS, RVLM, and PVN.4 Furthermore, subtype-specific expression has also been demonstrated in these autonomic nuclei, for example, ERβ seems to predominate in the PVN, whereas

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ERα is important in the NTS.7 An accurate understanding of how central estrogens protect against the development of hypertension depends directly on a more detailed knowledge of the precise role of both ER subtypes in different brain areas involved in BP regulation. Indeed, the specific roles of ERα and ERβ in BP regulation have been studied in mice harboring targeted deletions of ERα and ERβ and in animals with acute treatment of ER selective agonist. Estrogen activation of ERα, particularly that expressed centrally, is protective against the baroreceptor dysfunction and hypertension induced by angiotensin II in female mice.10,11 In recent microinjection studies on the effects of estrogen on BP, ERβ seems to mediate attenuated effects of estrogen on resting BP in the RVLM and glutamate-induced increase in BP in the PVN.7,12

Aldosterone (Aldo) is known to be an independent risk factor for cardiovascular disease,13 and the sites of action for Aldo pressor effects have been shown to include the CNS.14 Previously, we have demonstrated a clear role of the CNS in sex differences in the hypertensive effects of Aldo/salt.15 In these studies, central infusion of estrogen attenuated Aldo-induced hypertension in both males and ovariectomized (OVX) females, whereas central blockade of ERs by a nonselective antagonist augments Aldo pressor effects in intact females. Using selective ERα or ERβ agonist and methods to specifically silence ERα or ERβ expression in the PVN and RVLM, the present study further investigated regional specificity of different ER subtypes in the protective actions of estrogen in the female rat model of Aldo-induced hypertension.

Methods

Ninety-nine Sprague-Dawley female rats (10–12 weeks old, Harlan) were used. The rats were divided into 12 groups: (1–3) intracerebroventricular (icv) infusions of vehicle, a selective ERβ agonist diaryl-propiominitril (DPN) or a selective ERα agonist propyl-pyrazole-triol (PPT) in OVX females (n=6 per group); (4–6) icv injections of scrambled recombinant adeno-associated virus (AAV) carrying small interference (si) RNA (AAV-siRNA-scramble [SCM]), AAV-siRNA to silence ERα (AAV-siRNA-ERα), AAV-siRNA to silence ERβ (AAV-siRNA-ERβ) in intact females (n=5 each group); (7–9) PVN microinjections of siRNA-SCM, siRNA-ERα, or siRNA-ERβ in intact females (n=6 per group); (10–12) RVLM microinjections of siRNA-SCM, siRNA-ERα, or siRNA-ERβ in intact females (n=6 per group). All of the groups were infused subcutaneously with Aldo combined with 1% NaCl as the sole drinking fluid, and 1% NaCl intakes were measured daily. Six additional groups treated in the same way as group 7 to 12, but without physiological studies, were performed for assessments of mRNA expression of ERα or ERβ in the RVLM or PVN (n=5 per group).

For reactive oxygen species (ROS) imaging studies, neurons were collected from the PVN of 8-day-old rat pups from Sprague-Dawley mothers (Harlan). The cultured cells were divided into 4 groups: (1) incubation with siRNA-SCM and vehicle (control), (2) incubation of siRNA-SCM and Aldo, (3) incubation of siRNA-ERα and Aldo, and (4) incubation of siRNA-ERβ and Aldo.

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Iowa Animal Care and Use Committee.

Additional details for surgical procedures and experimental protocols are available in the online-only Data Supplement.

Data Analysis

Mean arterial pressure (MAP) and HR are presented as mean daily values. Difference scores for MAP and HR were calculated for each animal based on the mean of the 5-day baseline subtracted from the mean of the final 5 days of treatment. One-way ANOVAs for the experimental groups were then conducted on the mean values of calculated difference scores. After establishing a significant ANOVA, post hoc analyses were performed with Tukey multiple comparison tests between pairs of mean change scores. To test differences in the mean of the 5-day baseline versus the mean of the final 5 days of treatment, paired t tests were performed in animals within same group. The same statistical methods were used to analyze the changes in HR, 1% NaCl intake, or differences in Aldo-induced ROS production in neurons and mRNA expression of ERα or ERβ in the PVN or RVLM treated with siRNA-SCM, siRNA-ERα, or siRNA-ERβ. All data are expressed as mean±SE. Statistical significance was set at P<0.05.

Results

Effect of icv Infusions of Selective ER Agonist on Aldo/NaCl-Induced Hypertension in OVX Females

Baseline values for MAP and HR were comparable before and after icv ER agonist infusions across all groups of OVX females (not shown in Figure 1A). Twenty-one days of Aldo/NaCl treatment resulted in a 21.1±2.1 mm Hg increase in MAP in OVX females with icv vehicle infusions. Icv infusion of either PPT or DPN for 21 days significantly attenuated Aldo/NaCl-induced hypertension (Δ11.4±2.5 and Δ11.1±1.6 mm Hg, respectively; P<0.05; Figure 1A and 1B). Systemic Aldo infusions produced significant, comparable increases in 1% NaCl intake (P<0.05; Figure S1A in the online-only Data Supplement) and decreases in HR (Figure S1B) in all groups when compared with rats given Aldo vehicle alone (34.5±6.4 mL/d; not shown in Figure S1A).

Figure 1. Intracerebroventricular (icv) infusion of propyl-pyrazole-triol (PPT) or diarylpropionitrile (DPN) for 21 days attenuated Aldo/NaCl-induced hypertension in ovariectomized (OVX) rats. A, Daily mean arterial pressures (MAP) before and during systemic infusion of Aldo in icv vehicle, PPT or DPN-treated OVX rats. B, Average changes in MAP across days induced by Aldo infusion in all groups (*P<0.05 vs baseline; #P<0.05 vs icv vehicle plus Aldo infusion).
Effect of icv Injections of siRNA-SCM, siRNA-ERα, or siRNA-ERβ on Aldo/NaCl-Induced Hypertension in Intact Females

Baseline values for MAP and HR were comparable before and after icv siRNA microinjection in all intact female rats (not shown in Figure 2A). In icv siRNA-SCM–injected females, 21 days of Aldo/NaCl treatment resulted in a slight but significant increase in MAP (Δ5.7±1.9 mm Hg; P<0.05). Icv injections of either siRNA-ERα or siRNA-ERβ significantly augmented Aldo-induced increase in MAP (Δ14.3±1.5 and Δ20.5±2.1 mm Hg, respectively; P<0.05; Figure 2A and 2B). Systemic Aldo infusions also produced significant, comparable increases in 1% NaCl intake (Figure S2A) and decreases in HR (Figure S2B) in all groups.

Effect of Bilateral PVN Microinjections of siRNA-SCM, siRNA-ERα, or siRNA-ERβ on Aldo/NaCl-Induced Hypertension in Intact Females

To determine whether ERs within the PVN are involved in female resistance to Aldo/NaCl hypertension, the effects of siRNA-ERα or siRNA-ERβ were evaluated. Baseline values for MAP and HR were comparable before and after siRNA PVN microinjection (Table S1). Systemic Aldo produced a slight but significant increase in MAP in PVN siRNA-SCM–injected females (Δ7.8±1.3 mm Hg; P<0.05). Bilateral PVN microinjection of siRNA-ERβ significantly augmented pressor effect of Aldo (Δ16.1±1.3 mm Hg; P<0.05). However, microinjection of siRNA-ERα resulted in only a 11.2±1.8 mm Hg change in MAP that was not significantly different from the increase seen in siRNA-SCM–injected females (Figure 3A and 3B). Systemic Aldo infusions also produced significant but comparable increases in 1% NaCl intake (Figure S3A) and decreases in HR (Figure S3B) in all groups.

Effect of RVLM Microinjections of siRNA-SCM, siRNA-ERα, or siRNA-ERβ on Aldo/NaCl-Induced Hypertension in Intact Females

To determine whether ERs within the RVLM are involved in female resistance to Aldo/NaCl hypertension, the effects of siRNA-ERα and siRNA-ERβ were evaluated. Baseline values for MAP and HR were comparable before and after RVLM microinjection of siRNA. Systemic Aldo resulted in a slight but significant increase in MAP in RVLM siRNA-SCM–injected females (Δ7.7±0.9 mm Hg; P<0.05). Bilateral RVLM microinjection of siRNA-ERβ significantly augmented MAP induced by Aldo infusion (Δ16.1±2.7 mm Hg; P<0.05). However, microinjection of siRNA-ERα resulted in only an 8.1±2.9 mm Hg increase in MAP that was not significantly different from the increase in siRNA-SCM–injected females (Figure 4A and 4B). Systemic Aldo infusions also produced significant, but comparable, increases in 1% NaCl intake (Figure S4A) and decreases in HR (Figure S4B) in all groups.

Effects of Autonomic Blockade on BP

Figure 5 shows the decreases in BP with acute ganglionic blockade in all groups. The reduction in BP response to hexamethonium injection before central treatment and...
systemic infusion of Aldo was comparable. After 21 days of Aldo infusion, acute hexamethionium injection resulted in a greater reduction in BP in O VX rats with icv vehicle (−44.2±2.9 mm Hg; P<0.05; Figure 5A) as compared with icv DPN (−30.1±3.5 mm Hg) or PPT (−31.6±3.6 mm Hg). Likewise, a greater reduction in BP after acute hexamethionium was induced in intact females with icv, PVN, or RVLM injections of siRNA-ERβ (−42.3±2.1, −43.6±4.2, and −44.3±5.1 mm Hg, respectively; P<0.05). In contrast, the BP reductions induced by hexamethionium in intact females with siRNA-ERα microinjection into these sites were not different from those rats injected with siRNA-SCM (Figure 5B–5D).

**Localization and the Effects of AAV Delivery of siRNA on ERα and ERβ mRNA Expression**

The effect of viral delivery of siRNA to knockdown ERα and ERβ in the PVN and RVLM was verified by real-time polymerase chain reaction (RT-PCR) analysis. AAV-siRNA constructs contained a green fluorescent protein (GFP) construct that served as a marker indicating the site of delivery and the cells affected in the PVN and RVLM. Twenty-one days after PVN or RVLM injections of siRNA-ERβ (Figure 6A–a,b) or siRNA-ERα (Figure 6A–c,d), highly robust GFP expression was present in the PVN and RVLM, but not in the subfornical organ (SFO; Figure 6A–e) or the (AP; Figure 6A–f). The PVN injection spreads through the nucleus, and GFP was present in both parvo- and magnocelluar regions. To confirm effective silencing of ERα and ERβ in the PVN and RVLM with these viruses, we performed RT-PCR analyses on the micropunches taken from the PVN and RVLM. As shown in Figure 6B and 6C, ERα and ERβ mRNA levels were reduced by 44% to 74% in rats treated with siRNA-ERα or siRNA-ERβ when compared with rats treated with the control vector, siRNA-SCM.

**Effects of Knockdown ERα or ERβ on Aldo-Induced ROS Production in Cultured PVN Neurons**

Figure 7A shows representative dihydroethidium (DHE) fluorescent images of cultured PVN neurons with Aldo treatment. Overnight incubation of Aldo induced a 1.9-fold increase in ethidium intensity in siRNA-SCM–treated neurons (n=51; P<0.05) as compared with that seen in PVN neurons with Aldo vehicle incubation (n=25). Treatment with either siRNA-ERα or siRNA-ERβ significantly enhanced this Aldo-induced increase in ROS production (siRNA-ERα, 2.8-fold, n=85; siRNA-ERβ, 3.1-fold, n=90; P<0.05; Figure 7B).

**Discussion**

The main findings of this study are (1) both central ERα and ERβ activation played a protective role in the development of Aldo/NaCl hypertension in female rats, (2) PVN or RVLM knockdown of ERβ augmented Aldo/NaCl hypertension in female rats, (3) knockdown of ERα in either the PVN or the RVLM had no significant effect on the Aldo pressor effect, although Aldo induced an increased tendency for BP elevation after bilateral PVN knockdown of ERα, and (4) the brain regions responsible for the protective effects of estrogen interaction with ERα in the development of Aldo/NaCl hypertension in females still need to be determined. These results indicate that the effects of central estrogen are regional and receptor subtype specific for their protective effects against the development of Aldo/NaCl hypertension.
It is well established that ERα and ERβ mediate estrogen effects in the cardiovascular system. Each of these receptors is encoded by separate genes, ESR1 and ESR2, respectively. The observation that uterine atrophy occurred in ERα-deficient mice provided the first evidence for different functions of ERα and ERβ. Hypertension has been reported only in ERβ-deficient mice. Moreover, peripheral application of the ERβ agonist 8β-VE2, but not ERα agonist 16α-LE2, decreased elevated BP in spontaneously hypertensive rats. In contrast, activation of either ER subtype attenuates elevated BP level and cardiovascular remodeling in Aldo/salt-treated rats. Previous studies from our laboratory have demonstrated that estrogen activation of ERα, particularly that expressed centrally, is protective against the baroreceptor dysfunction and hypertension induced by angiotensin II in female mice. Together, these results suggest that the functional role of ERα in BP regulation is different depending on the cause or model of hypertension.

**Figure 6.** mRNA expression of estrogen receptor-α (ERα) or ERβ in the paraventricular nucleus (PVN) or rostroventrolateral medulla (RVLM) were significantly knocked down in intact females with siRNA-ERα or siRNA-ERβ microinjections as compared with those with siRNA-scramble (SCM) injections. A, Viral delivery of the transgene for green fluorescent protein (GFP) results in robust transgene expression localized to the PVN (a) or RVLM (c). b and d, Higher magnification of a portion of a and c, respectively. GFP expression could not be detected in the subfornical organ (SFO) (e) and area postrema (AP) (f) in the same animal indicating that the injected siRNA did not leak into the ventricle. B and C, Real-time polymerase chain reaction analysis of ERα or ERβ mRNA expressions in the PVN or RVLM from intact females receiving PVN or RVLM microinjections of siRNA-scramble (SCM), siRNA-ERα, or siRNA-ERβ (*P<0.05 vs intact females with siRNA-SCM injections).

**Figure 7.** Knockdown estrogen receptor-α (ERα) or ERβ enhanced Aldo-induced reactive oxygen species (ROS) production in neurons cultured from the paraventricular nucleus (PVN). A, Representative confocal images of dihydroethidium (DHE)-loaded neurons cultured from the PVN showing the effects of Aldo on ROS production in neurons treated with siRNA-scramble (SCM), siRNA-ERα or siRNA-ERβ. Green in the cytoplasm represents green fluorescent protein (GFP), a marker for virus transfection. Red in the cytoplasm represents DHE, a marker for ROS production. B, Summary of standardized emission intensity of DHE fluorescence in PVN neurons with overnight treatment of vehicle or Aldo. Neurons were treated with Aldo 24 h after treatment with siRNA-SCM, siRNA-ERα, or siRNA-ERβ. The change in ROS production was standardized by neurons treated with siRNA-scramble (SCM) and vehicle (*P<0.05 vs neurons with siRNA-SCM plus vehicle; #P<0.05 vs neurons with siRNA-SCM plus Aldo).
and that ERβ is a key regulatory factor of BP. The present study confirms and extends these previous studies by showing that central activation of either ERα or ERβ by icv chronic infusions of selective ER agonists attenuates the pressor effects in Aldo/NaCl-treated OVX female rats. In contrast, central blockade of both ER subtypes augments Aldo-induced pressor effects in intact females, suggesting that either central ERα or ERβ might mediate, at least in part, the protective effects of estrogen against Aldo/NaCl-induced hypertension. Moreover, in Aldo/salt-treated rats, the fall in BP in response to ganglionic blockade was less in those with icv infusions of selective ERα or ERβ agonist and was more in those with icv injections of siRNA for ERα or ERβ as compared with their respective controls. These observations add to the previously described studies by establishing the importance of both central ERα and ERβ in the actions against elevated sympathetic activity induced by Aldo.

In rats, studies have shown that ERβ is the predominant ER in the PVN, although ERα mRNA has also been found in this nucleus. Both ERα and ERβ mRNA, as well as protein, are expressed in the RVLM. In studies on immunogold back-labeled PVN neurons with projections to the RVLM, 50% of the cells were found to express ERβ. Thus, the PVN and RVLM may be ideal sites for interaction between ERs and mineralocorticoid receptor (MR) in the regulation of BP and sympathetic nerve activity. In the present study, it was not unexpected to demonstrate that both PVN and RVLM ERβ knockdown augmented Aldo pressor effects, supporting a role for ERβ activation in both the PVN and the RVLM in attenuating the pressor effects of Aldo. Surprisingly, Aldo only induced a slight tendency to increase BP after bilateral PVN knockdown of ERα. Knockdown of ERα in the RVLM had no significant effect. Indeed, recent studies have shown that microinjection of estrogen into the RVLM reduces BP and sympathetic nerve activity. Likewise, estrogen in the PVN attenuates glutamate-induced pressor effects. In both studies, only ERβ agonist mimicked the inhibitory effects of estrogen on these hemodynamic parameters, suggesting estrogen in the RVLM and PVN induces vasodepressor effects mainly via the ERβ-mediated mechanisms. Taken together, we interpret the data as suggestive of a predominant role for ERβ in the PVN and RVLM in mediating the protective effects of estrogen in the development of Aldo hypertension.

The results from the present study also raise the question as to which nucleus/nuclei ERα interacts with MR to play a protective effect in the development of Aldo-induced hypertension. The studies on specific characteristic and subtype-specific expression of ERs in the CNS have shown that ERα is important in the NTS, a nucleus with neurons which receive input from neurons carrying visceral information, and which, in turn, projects directly to the RVLM, PVN, etc. Microinjections of estrogen into the NTS induce increases in parasympathetic tone and baroreceptor sensitivity and a decrease in sympathetic activity in both male and female rats. In angiotensin II–infused rats, the intensity of ERα immunoreactivity increased exclusively in the commissural NTS, whereas neither the number nor the intensity of ERβ-labeled cells changed. Moreover, neurons that contain both MR and the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) have been identified in the NTS. Therefore, one could hypothesize that the NTS is a site where ERα interacts with MR to regulate BP. Another possible site where ERα interacts with MR is the lamina terminalis (LT), including the organum vasculosum of the LT, median preoptic nucleus, and SFO. LT structures are involved in both sensing and processing input derived from humoral factors (eg, Aldo and extracellular osmolality) and transmitting this information to the PVN, and thereby could contribute to the regulation of Aldo-induced salt appetite, sympathetic activity, and BP. It has also been shown that LT structures, especially the SFO, have substantial ERα. Therefore, ERα in the NTS or LT structures may be responsible for the protective effects of estrogen in the development of Aldo-induced hypertension. In the present study, in brains with icv injections of siRNAs, we found robust expression of GFP in several nuclei involved in cardiovascular regulation, including the SFO, PVN, RVLM, and NTS. These results may explain the effectiveness of icv, but not PVN/RVLM injections of ERα. In future studies this powerful tool can be used to chronically knockdown ERα in specific nuclei, such as the NTS or LT, to determine which brain areas are responsible for the interaction between estrogen and Aldo.

Zhang et al have reported that icv Aldo resulted in increased DHE staining (indicating oxidative stress) and Fra-like activity (indicating neuronal excitation) in neurons of the PVN along with increased sympathetic activity. Previous studies from our laboratory have also shown that icv infusion of apocynin, a NADPH oxidase inhibitor, attenuated systemic Aldo/salt-induced hypertension. Genetic silencing of either NOX2 or NOX4, 2 subunits of NADPH oxidase, with siRNAs in the PVN attenuated Aldo pressor effects. Together these results suggest that Aldo increases sympathetic nerve activity through increased oxidative stress in the hypothalamus. It has been shown that estrogen inhibits angiotensin II–induced hypertension and activation of SFO neurons via interactions with intracellular ROS production. In the present studies, we used confocal microscopy imaging with the fluorescent indicator DHE as an indicator to measure changes in ROS production induced by Aldo in cultured PVN neurons. We found that PVN neurons showed a significant increase in ROS production after overnight incubation with Aldo. The Aldo-induced ROS production was further increased by genetic silencing either ERα or ERβ with siRNAs in the absence of their ligand, estrogen. Recent studies have demonstrated that ERα- or ERβ-dependent activation, molecular regulation, and organ protection may occur in the absence of estrogen. Therefore, the present results suggest that a potential mechanism involved in female protection from Aldo-induced hypertension may involve ER inhibition of MR-induced increases in ROS.

A key feature of the present study that involved the use of AAV delivery of siRNA for selective silencing of ERα or ERβ in the PVN and RVLM was to explore a regional specific effect of these ER subtypes. In agreement with previous studies, icv injections of siRNA induced significant, but incomplete, silencing of ERα or ERβ within brain tissue by 44% to 74%. It should be noted that the incomplete silencing of ER, especially ERα, may still permit partial ER activation...
to attenuate the pressor effect of Aldo so that the part of augmented pressor effects by knockdown of ERs has been offset. Moreover, mRNA gene expression results do not always reflect the protein expression or activity levels. Nevertheless, ERβ knockdown in either the PVN or the RVLM resulted in significantly potentiating Aldo-induced hypertension, whereas knockdowns of ERα in these nuclei had no significant effect. In addition, the brain areas immediately adjacent to the PVN are the anterior hypothalamus area and ventromedial hypothalamic area. It has been shown that the ventromedial hypothalamic area is a sexually dimorphic group containing many ERs expressing neurons that have extensive projections to areas of the brain involved in autonomic regulation, such as the NTS and RVL.M. However, GFP expression in the hypothalamic area and ventromedial hypothalamic area was very low. siRNA injection into the PVN also did not leak into the third ventricle because there was no GFP expression in the SFO or area postrema. Therefore, it is not likely that the effect of blockade of PVN ERs or ERβ on the Aldo-induced pressor response was from antagonism of ERs in the ventromedial hypothalamic area or other nuclei involved in cardiovascular regulation.

**Perspectives**

Clinical and experimental data indicate important interactions between mineralocorticoid and ER function in cardiovascular diseases. Activation of both ER subtypes in the periphery and the CNS plays a protective role in the development of Aldo/salt-induced cardiovascular remodeling and hypertension. The present results indicate that both PVN and RVLM ERβ, but not ERα in these nuclei, contribute to the protective effects of estrogen against elevated sympathetic activity and the pressor effects during mineralocorticoid excess. This indicates that there is a regional functional specificity of different ER subtypes in the brain. However, the brain regions responsible for the protective effects of estrogen interaction with ERα in the development of Aldo-induced hypertension still need to be determined.

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**Disclosures**

None.

**References**


What Is New?
• These studies demonstrate that both paraventricular nucleus and rostroventrolateral medulla estrogen receptor-β (ERβ), but not ERα in these nuclei, contribute to the protective effects of estrogen against elevated sympathetic activity and the pressor effects during mineralocorticoid excess.

What Is Relevant?
• The demonstration of regional specificity of different ER subtypes in the central nervous system, especially the effect of ERβ in certain nuclei involved in BP regulation, indicates that non-feminizing ERβ agonists have a therapeutic potential in the treatment of some forms of hypertension.

Summary
The study indicates that the effects of central estrogen are regional and receptor subtype specific for their protective effects against the development of Aldo/NaCl hypertension.

Novelty and Significance
Estrogen Receptor-β in the Paraventricular Nucleus and Rostroventrolateral Medulla Plays an Essential Protective Role in Aldosterone/Salt-Induced Hypertension in Female Rats
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Estrogen receptor-beta (ERβ) in the PVN and RVLM plays an essential protective role in aldosterone/salt-induced hypertension in female rats

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Central estrogen receptor and Aldo-induced hypertension
Methods

Surgical Procedures

Animals. Ninety-nine Sprague-Dawley female rats (10–12 wk old, Harlan) were used. They were housed in temperature- and light-controlled animal quarters and were provided with rat chow (7013 NIH-31 modified rat diet, 0.25% NaCl) ad libitum.

Ovariectomy and telemetry probe implantation. Bilateral ovariectomy was performed in female rats. A single 2-3 cm dorsal midline incision was made in the skin and underlying muscles. The ovaries were isolated, tied-off with sterile suture and removed, and the incisions were closed. Ten days later, rats were chronically instrumented with telemetry probes (TA11-PA40, DSI) through the femoral artery for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR).

Chronic icv cannula and osmotic pump implantation: After baseline BP and HR recordings were made, the OVX rats were again anesthetized with ketamine-xylazine mixture, and the icv cannula with osmotic pump (model 2004, ALZET Brain Infusion Kits, Alzet Co.) was implanted into the right lateral ventricle (the coordinates 0.9 mm caudal, 1.5 mm lateral to bregma, and 4.5 mm below the skull surface) for chronic infusion of vehicle (10% DMSO in 0.9% saline), PPT (30 µg/kg/day) or DPN (30 µg/kg/d) for 21 days. At the same time, osmotic pumps (model 2004; Alzet) containing Aldo (750 ng/h, Sigma) were implanted subcutaneously in the back, and tap water was changed to 1% NaCl.

Icw, PVN or RVLM microinjection of AAV-siRNA : After baseline BP and HR recordings were made, the intact females were again anesthetized with ketamine-xylazine mixture for icw, PVN or RVLM microinjection of siRNA-SCM, siRNA-ERα, or siRNA-ERβ (1.3 x 10¹² genomic particles/mL; GeneDetect®; icw, 2 µL; PVN: 200 nL, -1.8 mm caudal, ±0.5 mm lateral to bregma, and 7.6 mm below the skull surface; RVLM: 200 nL, -13.0 mm caudal, ±2.0 mm lateral to bregma and 9.8 mm below the skull surface). The microinjections of siRNA were given over 30 second and the injector was kept in icw, the PVN or RVLM for two more minutes. At the same time, osmotic pumps (model 2004; Alzet) containing Aldo (750 ng/h, Sigma) were implanted subcutaneously in the back and tap water was changed to 1% NaCl.

PVN neuronal cultures. Primary neuronal cultures were established from the PVN of pre-weanling pups (8 days old, 8-10 pups per culture). Cells were cultured for 4 days in Dulbecco’s Modified Eagles Medium (DMEM): Ham’s F12 medium (1:1) supplemented with 10% FBS and 1% L-glutamine-penicillin-streptomycin.

Experimental Protocol

Measurement of BP and HR. All rats were allowed 7 days to recover from transmitter implantation surgery before any measurements were made. Thereafter, BP and HR were telemetrically recorded and stored with the Dataquest ART data acquisition system (DSI). MAP and HR were collected for 5 baseline days and then for 21 consecutive days during Aldo pump implantation. At the end of each experiment, animals were deeply anesthetized with pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde. The locations of the RVLM or PVN injections in histological material were verified by visualization of expression of the reporter gene GFP using confocal microscopy. In order to not damage these nuclei, the injected site was a little bit above the nuclei, so that siRNA spread down into the PVN
or RVLM. If injections were not in right place, the GFP spread densely to brain areas immediately adjacent to the PVN or RVLM, or siRNAs leaked into the brain ventricle, we defined it as “missed injection”. The animals with missed injections were excluded from analysis.

**Evaluation of BP responses to autonomic blockade.** The autonomic contribution to resting BP was assessed by administering the ganglionic blocker hexamethonium (30 mg/kg, ip). Ganglionic blockade was repeated twice, once during baseline and then after 21 days of Aldo infusion. On the day of ganglionic blockade experiments, BP was recorded for 20 min both before and after hexamethonium injection. After hexamethonium injection, the largest decrease in BP occurred within 5 min. This nadir (2-3 min) was recorded as the maximum fall in BP.

**Measurement of mRNA Expression in the RVLM and PVN:** Total RNA was isolated from RLVM and PVN using Trizol method (Invitrogen) and treated with DNase I (Invitrogen). RNA integrity was checked by gel electrophoresis. Total RNA was reverse transcribed using random hexamers following the manufacturer’s instructions (Applied Biosystems). Real time PCR was conducted using 200-300 ng of cDNA and 500 nM of each primer in a 20 µl reaction with iQ SYBR Green Supermix (Bio-Rad). Amplification cycles were conducted at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and annealing/extension at 60°C for 30 s. Reactions were performed in duplicate and analyzed using a C1000 thermocycler system (Bio-Rad). Samples that did not yield homogenous melt curves were excluded. Changes in mRNA expression levels were normalized to GAPDH levels and calculated using the ΔΔCt method. Results are expressed as relative fold change, mean of fold change ± SE. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of the primers are shown as following, ERα (accession # AB477039, 128bp), forward primer: 5′-CAT CGA TAA GAA CCG GAG GA-3′; Reverse primer: 5′-TCT GAC GCT TGT GCT TCA AC-3′. ERβ (accession # AB190770, 112bp), forward primer: 5′-GAA GCT GAA CCA CCC AAT GT-3′; Reverse primer: 5′-CAA TCA TGT GCA CCA GTT CC-3′.

**Measurement of ROS production in cultured PVN neurons.** After incubating cells for 24 h under serum free conditions, the cultured neurons were treated with vehicle, siRNA-SCM, siRNA-ERα, or siRNA-ERβ for another 24 hours followed by overnight incubation with Aldo (10 µM) in normal serum condition. On the experimental day, the neurons were loaded with dihydroethidium (DHE, 5µM, Invitrogen) for 30 minutes, and then ethidium fluorescence was imaged using confocal microscopy. Neurons are roundish, domed cells with one or two thin processes as verified by terminal experimental KCl-induced calcium flux. DHE fluorescence of neurons was quantified using Fluoview 5 (Olympus, Japan) analysis software and expressed relative to fluorescence of vehicle treated neurons.
**Table S1.** Baseline mean arterial pressure (MAP) and heart rate (HR) in all groups.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX icv Vehicle</td>
<td>104.7±2.1</td>
<td>367.7±2.2</td>
</tr>
<tr>
<td>OVX icv PPT</td>
<td>106.4±3.5</td>
<td>360.2±2.8</td>
</tr>
<tr>
<td>OVX icv DPN</td>
<td>105.3±1.7</td>
<td>355.0±3.9</td>
</tr>
<tr>
<td>Icv siRNA-SCM</td>
<td>101.3±1.8</td>
<td>370.1±7.3</td>
</tr>
<tr>
<td>Icv siRNA-ERα</td>
<td>102.8±2.3</td>
<td>372.2±3.2</td>
</tr>
<tr>
<td>Icv siRNA-ERβ</td>
<td>103.5±3.0</td>
<td>370.0±6.5</td>
</tr>
<tr>
<td>PVN siRNA-SCM</td>
<td>103.0±2.2</td>
<td>379.1±7.7</td>
</tr>
<tr>
<td>PVN siRNA-ERα</td>
<td>101.8±3.5</td>
<td>357.1±8.7</td>
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<tr>
<td>PVN siRNA-ERβ</td>
<td>101.5±2.4</td>
<td>367.7±5.6</td>
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<tr>
<td>RVLM siRNA-SCM</td>
<td>102.3±1.7</td>
<td>380.1±8.0</td>
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<tr>
<td>RVLM siRNA-ERα</td>
<td>103.6±2.4</td>
<td>372.9±10.5</td>
</tr>
<tr>
<td>RVLM siRNA-ERβ</td>
<td>102.5±1.9</td>
<td>371.5±6.2</td>
</tr>
</tbody>
</table>

**Table S2.** Changes in body weight (BW) after central administration of estrogen receptor (ER) agonists or siRNA for ERs plus peripheral aldosterone infusion in all groups (*p<0.05 vs baseline BW of intact females; #p<0.05 vs BW changes in groups with central injection of ER agonist or siRNA; †p<0.05 vs BW changes in ovariectomized (OVX) icv vehicle and intact groups with central injection of siRNAs).

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Baseline (g)</th>
<th>After treatment</th>
<th>Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX icv Vehicle</td>
<td>272.5±8.9*</td>
<td>311.5±10.1</td>
<td>39.0±5.2#</td>
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<tr>
<td>OVX icv PPT</td>
<td>278.3±2.4*</td>
<td>272.5±5.9</td>
<td>-5.8±5.6†</td>
</tr>
<tr>
<td>OVX icv DPN</td>
<td>282.3±6.5*</td>
<td>284.0±5.7</td>
<td>1.8±5.6†</td>
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<tr>
<td>Icv siRNA-SCM</td>
<td>245.7±6.5</td>
<td>262.3±4.2</td>
<td>17.2±3.5</td>
</tr>
<tr>
<td>Icv siRNA-ERα</td>
<td>231.8±5.2</td>
<td>246.7±3.9</td>
<td>14.8±2.9</td>
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<tr>
<td>Icv siRNA-ERβ</td>
<td>239.1±4.7</td>
<td>255.3±3.8</td>
<td>15.9±3.2</td>
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<tr>
<td>PVN siRNA-SCM</td>
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<td>274.1±6.5</td>
<td>18.5±2.5</td>
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<tr>
<td>PVN siRNA-ERα</td>
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<td>261.5±4.7</td>
<td>16.8±3.5</td>
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<tr>
<td>PVN siRNA-ERβ</td>
<td>236.0±5.5</td>
<td>251.5±3.2</td>
<td>15.5±2.6</td>
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<tr>
<td>RVLM siRNA-SCM</td>
<td>232.3±6.5</td>
<td>249.5±6.8</td>
<td>17.3±2.1</td>
</tr>
<tr>
<td>RVLM siRNA-ERα</td>
<td>232.5±4.5</td>
<td>245.8±3.8</td>
<td>13.5±2.6</td>
</tr>
<tr>
<td>RVLM siRNA-ERβ</td>
<td>235.6±5.1</td>
<td>250.8±3.2</td>
<td>15.1±3.7</td>
</tr>
</tbody>
</table>
**Figure S1.** Systemic Aldosterone (Aldo) infusions produced significant, comparable increases in 1% NaCl intake and decreases in heart rate (HR) in ovariectomized (OVX) female rats treated with central vehicle, PPT or DPN. 

**A.** Mean daily 1% NaCl intake during chronic Aldo infusion. 

**B.** Average changes in HR across days induced by Aldo infusion.
Figure S2. Systemic Aldosterone (Aldo) infusions produced significant, comparable increases in 1% NaCl intake and decreases in heart rate (HR) in female rats with icv injections of siRNA-SCM, siRNA-ERα, or siRNA-ERβ. A. Mean daily 1% NaCl intake during chronic Aldo infusion. B. Average changes in HR across days induced by Aldo infusion.
Figure S3. Systemic Aldosterone (Aldo) infusions produced significant, comparable increases in 1% NaCl intake and decreases in heart rate (HR) in female rats with PVN microinjections of siRNA-SCM, siRNA-ERα, or siRNA-ERβ. A. Mean daily 1% NaCl intake during chronic Aldo infusion. B. Average changes in HR across days induced by Aldo infusion.
**Figure S4.** Systemic Aldosterone (Aldo) infusions produced significant, comparable increases in 1% NaCl intake and decreases in heart rate (HR) in female rats with RVLM microinjections of siRNA-SCM, siRNA-ERα, or siRNA-ERβ. **A.** Mean daily 1% NaCl intake during chronic Aldo infusion. **B.** Average changes in HR across days induced by Aldo infusion.
Figure S5. Icv injection of AAV-siRNAs results in robust transgene expression displayed by green fluorescent protein (GFP) in the SFO, PVN, RVLM and NTS.
**Fig. S6**

Figure S6 shows the green fluorescent protein (GFP) expression of AAV-siRNAs in missed injection sites of the RVLM (A) and PVN (B). Arrows, injected sites; py, pyramidal tract; LPGi, lateral paragigantocellular nucleus; RVL, rostroventrolateral reticular nucleus; 3V, the third ventricle; PVN, paraventricular nucleus.