Ovariectomy Augments Hypertension Through Rho-Kinase Activation in the Brain Stem in Female Spontaneously Hypertensive Rats

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Abstract—Estrogen protects against increases in arterial pressure (AP) by acting on blood vessels and on cardiovascular centers in the brain. The mechanisms underlying the effects of estrogen in the brain stem, however, are not clear. The aim of the present study was to determine whether ovariectomy affects AP via the Rho/Rho-kinase pathway in the brain stem. We performed bilateral ovariectomy in 12-week-old female spontaneously hypertensive rats. AP and heart rate (HR), measured using radiotelemetry in awake rats, were increased in ovariectomized rats compared with control rats (mean AP: 163±3 versus 144±4 mm Hg; HR: 455±4 versus 380±6 bpm). Continuous intracisternal infusion of Y-27632 significantly attenuated the ovariectomy-induced increase in AP and HR (mean AP: 137±6 versus 163±3 mm Hg; HR: 379±10 versus 455±4 bpm). In addition, we confirmed the increase of Rho-kinase activity in the brain stem in ovariectomized rats, and the increase was attenuated by intracisternal infusion of Y-27632 via the phosphorylated ezrin, radixin, and moesin (ERM) family, which are Rho-kinase target proteins. Furthermore, angiotensin II type 1 receptor expression in the brain stem was significantly greater in ovariectomized rats than in control rats, and the increase was partially reduced by intracisternal infusion of Y-27632.

The incidence of cardiovascular diseases is lower in premenopausal women than in age-matched men1–3 and postmenopausal women.4 The decreased protective effect against cardiovascular diseases, such as hypertension, in postmenopausal women is thought to be because of endogenous ovarian estrogen depletion. Estrogen decreases arterial pressure through direct effects on blood vessels5,6 and through effects on central cardiovascular regulatory centers by modulating autonomic function of the cardiovascular system.7,8 Hormone replacement therapy in postmenopausal women favorably affects cardiovascular regulation by improving baroreflex function and heart rate (HR) variability (HRV)9 and by decreasing sympathetic nerve activity.9 In the central nervous system (CNS), endogenous estrogen has numerous effects through estrogen receptor–dependent and -independent pathways.5,11 Estrogen and estrogen receptors are present in the brain stem where the vasomotor centers, such as the nucleus tractus solitarius (NTS) and the ventrolateral medulla, are located.12 Medullary injections of exogenous estrogen decrease arterial pressure, HR, and renal sympathetic nerve activity and enhance reflex control of the HR in male rats, as well as in ovariectomized female rats,13 suggesting that estrogen has beneficial effects on autonomic functions.14

Rho-Kinase is a serine–threonine protein kinase and is one of the effectors of the small GTP-binding protein Rho. This pathway is involved in various cellular functions including smooth muscle contraction, actin cytoskeleton organization, cell proliferation, and cell motility.15–18 In vascular smooth muscle cells, activation of this pathway contributes to the pathophysiology of hypertension via smooth muscle contraction.19,20 In the CNS, the Rho/Rho-kinase pathway contributes to the formation of dendritic spines.21 Dendritic spines form the postsynaptic contact sites of excitatory synapses in the CNS22 and are thought to be involved in synaptic transmission.23 We reported previously that Rho-kinase in the brain stem modulates glutamate sensitivity24 and maintains arterial pressure via the sympathetic nervous system and that activation of the Rho/Rho-kinase pathway might contribute to the central mechanisms of hypertension.25,26 Estrogen also regulates the formation of excitatory synapses on dendritic spines.27 Estrogen treatment increases spine number and...
synaptic density on apical and basal dendrites of CA1 pyramidal neurons in ovariectomized adult female rats. These findings led to the hypothesis that the effects of endogenous estrogen on central cardiovascular regulation involve alterations in Rho-kinase activity in the central cardiovascular center. Therefore, the aim of the present study was to determine whether the depletion of endogenous estrogen affects arterial pressure via the Rho/Rho-kinase pathway in the brain stem.

For this purpose, we performed a bilateral ovariectomy in 12-week-old female spontaneously hypertensive rats (SHRs). Y-27632, a specific Rho-kinase inhibitor, was then infused intracisternally for 2 weeks with a miniosmotic pump. Arterial pressure and HR were measured in awake rats using a radiotelemetry system. In a separate group of animals, we confirmed that serum and cerebrospinal fluid (CSF) 17β-estradiol concentrations were decreased in ovariectomized rats. We then compared the Rho-kinase activity in the brain stem between control rats and ovariectomized rats. Finally, we compared the expression level of angiotensin II type 1 receptors (AT1Rs) in the brain stem between control rats and ovariectomized rats, because estrogen functions upstream of the renin–angiotensin system and the Rho/Rho-kinase pathway is downstream of the renin–angiotensin system.

Methods

This study was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and was conducted according to the Guidelines for Animal Experiments of Kyushu University. Female SHRs (11-week-old, SLC Japan, Hamamatsu, Japan) were used in the present study. A flexible catheter containing an arterial pressure transmitter was introduced into the abdominal aorta just below the renal arteries under sodium pentobarbital (50 mg/kg IP) anesthesia. After surgery, rats were housed singly in cages and allowed unrestricted movement. Rats were fed food devoid of phytoestrogens (AIN 76A, Kyudo Co, Ltd). For 21 days after ovariectomy, arterial pressure and HR were recorded continuously for 10 minutes every other day using a UA-10 telemetry system (Data Sciences International).

Surgical Procedures

Seven days after catheter implantation, we performed bilateral ovariectomy (OVX) or sham operation (control) in the then 12-week-old rats under sodium pentobarbital (50 mg/kg IP) anesthesia. Ovariectomized rats were further randomly divided into 2 groups. The first group received continuous infusion of vehicle (a-CSF, 0.25 μL/h; OVX-VEH rats), and the second group received continuous infusion of Y-27632, a specific Rho-kinase inhibitor (5 mmol/L Y-27632, 0.25 μL/h; OVX-Y rats), intracisternally for 2 weeks via a miniosmotic pump (Alzet model 1002; Durect Corp.). The miniosmotic pump, filled with vehicle or Y-27632, was implanted subcutaneously in the back and connected to a polyethylene tube (PE 10). A small hole was then made in the atlantooccipital membrane, which covers the dorsal surface of the medulla, and the tip of the tube was placed intracisternally and fixed in place with tissue adhesive. After surgery, the rats were free to move about their cages. The infusion was calculated to last 14 days.

Power Spectral Analysis

To evaluate sympathetic activity, we performed power spectral analysis on day 11 after OVX or sham operation. HR was recorded from 20-minute ECGs performed in awake rats using the radiotelemetry system. The ECG data were calculated using the Powerlab system and Chart 4 software (AD Instruments), and the power spectra of the R-R interval was calculated using the maximum entropy method with MemCalc software (Suwa Trust Co, Ltd). The frequency bands were adapted for analysis in rats: a very low frequency band (VLF) of 0 to 0.25 Hz, a low frequency band (LF) of 0.25 to 0.8 Hz, and a high frequency band (HF) of 0.8 to 2.4 Hz.

Western Blot Analysis

The animals of each group (control rats, OVX-VEH rats, and OVX-Y rats) were killed with an overdose of sodium pentobarbital on day 11 after OVX or sham operation, and whole brain stem tissues were obtained. The animals used in this experiment were different from those in which arterial pressure, HR, and ECG were monitored. The tissues were obtained as the whole brain stem to ensure that the same areas from each animal were used and then homogenized in lysing buffer containing 40 mmol/L HEPES, 1% Triton X-100, 10% glycerol, 1 mmol/L Na3VO4, and 1 mmol/L phenylmethylsulfonyl fluoride. The tissue lysate was centrifuged, and the supernatant was collected. The protein concentration was determined using a BCA protein assay kit (Pierce Chemical). A protein aliquot (15 μg) from each sample was separated on a 10% sodium dodecyl sulfate–polyacrylamide gel and subsequently transferred onto polyvinylidene fluoride membranes (Immobilon-P membrane, Millipore). Membranes were incubated with rabbit anti-phosphorylated ERK proteins [ezrin (Thr567), radixin (Thr564), and moesin (Thr558)], which are the target proteins of Rho-kinase (this primary antibody was made by Kaibuchi and colleagues and has been characterized and used in many previous reports). Membranes were then incubated with a horseradish peroxidase-conjugated horse anti-rabbit IgG antibody (1:10 000). Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit, Amersham Pharmacia Biotechnology), and the film was analyzed using NIH Image (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Western blot analysis for AT1R was performed as described above using rabbit anti-AT1R antibody (1:1000, Santa Cruz Biotechnology).

Measurement of Estradiol Concentration in the Serum and CSF

A separate set of 12-week-old female SHRs was divided into 2 groups. In the first group, bilateral OVX alone was performed (OVX rats). In the second group, sham operation was performed (Sham rats). The animals in each group were anesthetized with an overdose of sodium pentobarbital on day 11 after the intervention, and blood samples from the femoral vein were obtained to measure the serum 17β-estradiol concentration by radioimmunoassay performed by SRL Inc. Furthermore, a small hole was made in the atlantooccipital membrane, the tip of a tube connected to a syringe was placed intracisternally, and CSF was collected. Because only a small amount of CSF could be collected from each animal, samples from 5 animals of each group were pooled, and the 17β-estradiol concentration was measured.

Statistical Analysis

All of the values are expressed as mean±SEM. Two-way ANOVA was used to compare differences in mean arterial pressure (MAP) and HR between the Y-27632 and vehicle infusion groups. Comparisons between any mean values were performed by application of Bonferroni’s correction for multiple comparisons. An unpaired t test was used to compare the baseline values and the effects of each intervention between groups. Differences were considered to be statistically significant when P<0.05.

Results

Effects of OVX and Intracisternal Infusion of Y-27632 on Arterial Pressure and HR Measured by Radiotelemetry

The time course of MAP and HR after OVX and intracisternal infusion of Y-27632 is shown in Figure 1. The baseline
values of arterial pressure in control rats, OVX-VEH rats, and OVX-Y rats were 192\pm 7, 189\pm 9, and 187\pm 7 mm Hg (systolic arterial pressure); 115\pm 3, 113\pm 2, and 112\pm 4 mm Hg (diastolic arterial pressure); and 142\pm 3, 138\pm 2, and 137\pm 3 mm Hg (MAP), respectively. The baseline values of HR in control rats, OVX-VEH rats, and OVX-Y rats were 373\pm 7, 372\pm 6, and 362\pm 7 bpm, respectively. Arterial pressure and HR were significantly increased in OVX-VEH rats. Y-27632 significantly attenuated the increase in arterial pressure and HR in OVX-Y rats. After discontinuing treatment with Y-27632, arterial pressure and HR increased to levels similar to those in OVX-VEH rats. In control rats, arterial pressure and HR did not change after the operation.

Effects of OVX and Intracisternal Infusion of Y-27632 on HR Power Spectra
The effects of OVX on HR power spectra are shown in Figure 2. The VLF and LF components were significantly increased in OVX-VEH rats. The VLF and LF components in OVX-Y rats were 373\pm 7, 372\pm 6, and 362\pm 7 bpm, respectively. Arterial pressure and HR were significantly increased in OVX-VEH rats. Y-27632 significantly attenuated the increase in arterial pressure and HR in OVX-Y rats. After discontinuing treatment with Y-27632, arterial pressure and HR increased to levels similar to those in OVX-VEH rats. In control rats, arterial pressure and HR did not change after the operation.

Western Blot Analysis of Rho-Kinase Activity
The extent of ERM phosphorylation, which represents Rho-kinase activity, was greater in OVX-VEH rats and in OVX-Y rats than in control rats (Figure 3A). The increase in ERM phosphorylation in OVX-Y rats, however, was significantly less than that in OVX-VEH rats. The expression level of the total ERM family did not differ between groups.

AT1R Expression Level
The AT1R expression level was significantly increased in OVX-VEH rats and OVX-Y rats compared with control rats (Figure 3B). The increase in AT1R expression in OVX-Y rats was significantly smaller than that in OVX-VEH rats (Figure 3B).

Effects of OVX on Serum and CSF Estradiol Concentrations
On day 11 after the intervention, the serum 17\beta-estradiol concentration was decreased in OVX rats compared with Sham rats (139\pm 51 pg/mL versus 34\pm 11 pg/mL; n=5 for each; P<0.05). In addition, the CSF 17\beta-estradiol concentration was also decreased in OVX rats (270.0 pg/mL versus 61.5 pg/mL).

Figure 1. Chronic intracisternal infusion of Y-27632. Time course of MAP and HR after ovariectomy with continuous infusion of Y-27632 or vehicle intracisternally for 2 weeks with a miniosmotic pump. MAP and HR were increased in OVX-VEH rats. Y-27632 significantly attenuated the increase in MAP and HR in OVX-Y rats (n=4 for each). *P<0.05 vs control rats. #P<0.05 vs OVX-VEH rats.

Figure 2. Evaluation of sympathetic nerve activity by power spectral analysis. Each graph shows the power density of each spectrum. The power density of VLF and LF reflects sympathetic nerve activity, and the ratio of LF/HF reflects the sympathovagal balance. Both parameters increased in OVX+VEH rats compared with control rats (n=5 for each). *P<0.05 vs control rats. #P<0.05 vs OVX+VEH rats.
The present study demonstrated that in female SHRs, OVX induced an increase in arterial pressure via activation of the sympathetic nervous system, attributable, at least in part, to Rho-kinase activation in the brain stem. Furthermore, the results of the present study suggest that angiotensin II in the brain stem contributes to these mechanisms.

We used only female SHRs as a hypertensive model in this study. Previous studies focusing on the interaction between cardiovascular regulation and estrogen demonstrated that OVX has no effect on arterial pressure in normotensive animals. Although the Rho-kinase activity and renin–angiotensin system in the brain stem might be affected by OVX in normotensive rats, the effects of OVX, like in postmenopausal women, are more obvious in hypertensive rats than in normotensive rats. Therefore, we used only SHRs in the present study. Previous studies also indicated that OVX alters arterial pressure only in salt-sensitive hypertensive models, such as SHRs and Dahl salt-sensitive rats. There are reports that bilateral OVX in hypertensive rats enhances salt sensitivity but only increases arterial pressure in rats fed a high NaCl diet. In the present study, arterial pressure and HR were significantly increased in OVX-VEH rats fed a standard NaCl diet compared with controls from day 7 after bilateral OVX. The discrepant results might be because of differences in age at the time of OVX or in the diet. In fact, rats ovariectomized at a young age and fed a normal diet only have a significant increase in arterial pressure when fed a high-NaCl diet, whereas rats ovariectomized at an adult age and fed a phytoestrogen-devoid diet have increased arterial pressure under both a standard NaCl diet and a high-NaCl diet. Furthermore, we measured arterial pressure by radiotelemetry. This system allowed us to measure arterial pressure in awake rats under relatively stress-free conditions compared with other methods, such as the tail-cuff method. In a preliminary study, when we measured arterial pressure by the tail-cuff method, OVX tended to increase arterial pressure, but the difference was not significant (data not shown). These methodologic differences might also cause discrepant results from previous studies. In fact, arterial pressure measured by radiotelemetry is significantly increased in ovariec-

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** A, Western blot analysis demonstrating p-ERM expression in the brain stem. The p-ERM expression level was significantly higher in OVX-VEH rats than in control rats, and its increase was significantly attenuated by Y-27632 infusion. The data are expressed as the relative ratio to controls. The ratio of phosphorylated/total ERM family expression in control rats was assigned a value of 1 (n=5 for each). **P<0.01, *P<0.05 vs control rats. #P<0.05 vs OVX+VEH rats. B, Western blot analysis demonstrating AT1R expression in the brain stem. The AT1R expression level was significantly higher in OVX-VEH rats than in control rats, and its increase was significantly attenuated by Y-27632 infusion. The data are expressed as relative ratio to control. The extent of the AT1R in control rats was assigned a value of 1 (n=4 for each). **P<0.01, *P<0.05 vs control rats. #P<0.05 vs OVX+VEH rats.

Intracisternal infusion of Y-27632 prevented the OVX-induced increase in arterial pressure, suggesting that endogenous Rho-kinase in the brain stem has an important role in OVX-induced hypertension in female SHRs. The concentration of Y-27632 (5 mmol/L) used in the present study has selective effects on the CNS. Intracisternal drug infusion affects neurons in several regions of the autonomic cardiovascular area. We demonstrated previously, however, that the Rho/Rho-kinase pathway is activated in the NTS of SHRs but not normotensive rats, and Rho-kinase inhibition in the NTS induces a significant reduction in arterial pressure. In addition, the NTS is positioned at the dorsal surface of the medulla. Therefore, Y-27632 might have greater effects on neurons of the NTS than on neurons of other nuclei, although we cannot exclude the possibility that it affects other autonomic areas in the brain stem. Together, these findings suggest that the effect of Y-27632 is mediated in part by the inhibition of Rho-kinase activity in the NTS.

In the present study, it is unlikely that the results are because of nonspecific effects caused by the surgical procedure, because continuous intracisternal infusion of vehicle using the same device did not suppress the increase in arterial pressure, and after discontinuing the Y-27632 infusion, arterial pressure increased to levels similar to those in OVX-VEH rats. In addition, ERM phosphorylation in the brain stem was significantly increased in OVX-VEH rats and reduced in OVX-Y rats, which strongly suggests that the Rho-kinase activity in the brain stem of OVX-VEH rats was increased and that Y-27632 suppressed Rho-kinase activity. We estimated the extent of ERM phosphorylation as a marker of Rho-kinase activity. The ERM family members are concentrated in the actin-rich cell surface, cross-link actin filaments with the plasma membrane, and contribute to cell–cell adhesion and maintenance of cell shape and cell motility. Although the role of ERM family members in the central cardiovascular regulation is not clear, and ERM family members might be the substrates of other kinases, ERM phosphorylation is commonly used as an indicator of Rho-kinase activity.
In the present study, intracisternal infusion of Y-27632 nearly abolished the OVX-induced increase in arterial pressure. We reported previously that Rho-kinase activity in the brain stem of hypertensive rats is increased compared with normotensive rats.\textsuperscript{25,26} Therefore, intracisternal infusion of Y-27632 might suppress not only the OVX-induced increase in Rho-kinase activity but also basal Rho-kinase activity, thereby inducing a greater reduction in arterial pressure than that induced by OVX. In most previous studies, arterial pressure changes were evaluated several weeks after OVX. In the present study, \textapprox 1 week after OVX, arterial pressure of ovarioctomized rats was significantly increased compared with that of control rats. As mentioned above, we used more sensitive methods for measuring arterial pressure. In addition, we confirmed that the 17\textbeta-estradiol concentration, both in the serum and the CSF, decreased by \textapprox 25\% at 11 days after OVX. Although the serum and the CSF estradiol concentrations were markedly decreased by OVX, the concentrations were still high. We speculate that other organs, such as adrenal glands or adipocytes, produced estradiol after OVX. We did not address these issues, however, and do not have precise interpretations for this finding. In the present study, we primarily wanted to confirm that OVX reduced serum and CSF estradiol concentrations.

We estimated sympathetic activity using the HRV power spectral analysis. HRV is used as a noninvasive marker of autonomic outflow to the heart in a variety of disease states.\textsuperscript{50} HRV has a very specific pattern in the frequency domains delineated by the HF, LF, and VLF components.\textsuperscript{36} Spontaneous VLF power, which is 0 to 0.25 Hz, and LF power, which is 0.25 to 0.8 Hz, in the rat are particularly related to sympathetic nerve activity.\textsuperscript{36–38} In fact, an increased LF component in R–R variability occurs in various conditions known to decrease baroreflex gain and increase sympathetic outflow, such as tilt, mental stress, and exercise.\textsuperscript{51} On the other hand, the HF component is attributed to vagal and respiratory control, and the LF/HF ratio is used as an index of sympathovagal balance.\textsuperscript{52} Therefore, we also used spectral analysis of the HR in the present study. The VLF and LF powers or the LF/HF ratio were greater in OVX-VEH rats than in control rats, and those in OVX-Y rats were significantly reduced compared with OVX-VEH rats. Furthermore, the HR increase could also be an indicator of activation of the sympathetic nervous system. In the present study, HR was greater in OVX-VEH rats than in control rats and that in OVX-Y rats was significantly reduced compared with OVX-VEH rats. These results indicate that sympathetic activity was increased by OVX, and intracisternal infusion of Y-27632 significantly attenuated the increase in sympathetic activity.

Estrogen decreases arterial pressure by acting on blood vessels or the kidney via the AT1R.\textsuperscript{5,6,41} Furthermore, the renin–angiotensin system is also a major pathway of the central mechanisms of hypertension. Previous reports suggest that angiotensin II contributes to the neural mechanisms of hypertension.\textsuperscript{53,54} In addition, inhibition of Rho-kinase activity suppresses angiotensin II–induced cardiovascular effects.\textsuperscript{34} Therefore, we examined AT1R expression levels in each group to address the possibility of a partial interaction between the renin–angiotensin system and the Rho/Rho-kinase pathway in OVX-induced hypertension. The AT1R levels in the brain stem were significantly increased by OVX, and this increase was attenuated by intracisternal infusion of Y-27632. These results suggested that angiotensin II in the brain stem contributes to the mechanisms of OVX-induced hypertension in female SHRs. The Rho/Rho-kinase pathway is downstream of the renin–angiotensin system.\textsuperscript{34,35} RhoA regulates the expression of AT1R,\textsuperscript{59} however, and Y-27632 inhibits not only Rho-kinase activity, but also RhoA activity.\textsuperscript{56} Therefore, Y-27632 might attenuate RhoA activity by direct effects or negative feedback mechanisms of the Rho/Rho-kinase pathway and, thus, lead to the inhibition of AT1R expression. The finding that Y-27632 had only a weak effect on AT1R expression, together with the results of previous studies,\textsuperscript{34,35} suggest that the depletion of endogenous estrogen activates the Rho/Rho-kinase pathway in the brain stem and might also activate the renin–angiotensin system.

In conclusion, we demonstrated that the depletion of endogenous estrogen by OVX increases arterial pressure in female SHRs, at least in part, via activation of the renin–angiotensin system and Rho/Rho-kinase pathway in the brain stem.

**Perspectives**

It is not known how OVX induces increases in arterial pressure in female SHRs. In the CNS, both the Rho/Rho-kinase pathway and estrogen regulate the formation of excitatory synapses on dendritic spines.\textsuperscript{27} Dendritic spines form the postsynaptic contact sites of excitatory synapses in the CNS\textsuperscript{22} and are associated with glutamate sensitivity.\textsuperscript{23} Therefore, estrogen depletion might induce morphological or functional changes in the dendritic spines via Rho-kinase activation. On the other hand, estrogen depletion increases arterial pressure and hypothalamic norepinephrine levels,\textsuperscript{46} and hypothalamus neurons project to the dorsomedial medulla neurons, such as those in the NTS.\textsuperscript{57} These findings suggest that estrogen depletion affects dorsomedial medulla neurons via changes in the hypothalamic norepinephrine levels. Although further studies are needed to clarify the mechanisms of these effects, the Rho/Rho-kinase pathway in the brain stem might be involved in the mechanisms underlying OVX-induced hypertension, because Rho-kinase in the NTS is involved in central mechanisms of cardiovascular regulation via modulation of the sensitivity of NTS neurons to glutamate.\textsuperscript{24–26,58}

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**Disclosures**

None.

**References**


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