Extracellular Signal–Regulated Kinase 1/2 Activation, via Downregulation of Mitogen-Activated Protein Kinase Phosphatase 1, Mediates Sex Differences in Desoxycorticosterone Acetate-Salt Hypertension Vascular Reactivity

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Abstract—Extracellular signal–regulated kinase (ERK)1/2 has been reported to play a role in vascular dysfunction associated with mineralocorticoid hypertension. We hypothesized that, compared with female rats, an upregulation of ERK1/2 signaling in the vasculature of male rats contributes to augmented contractile responses in mineralocorticoid hypertension. Uninephrectomized male and female Sprague-Dawley rats received desoxycorticosterone acetate (DOCA) pellets (200 mg per animal) and saline to drink for 3 weeks. Control uninephrectomized rats received tap water to drink. Blood pressure, measured by telemetry, was significantly higher in male DOCA rats (191±3 mm Hg) compared with female DOCA rats (172±7 mm Hg; n=5). DOCA treatment resulted in augmented contractile responses to phenylephrine in aorta (22±3 mN; n=6) and small mesenteric arteries (13±2 mN; n=6) from male DOCA rats versus uninephrectomized male rats (16±3 and 10±2 mN, respectively; P<0.05) and female DOCA rats (15±1 and 11±1 mN, respectively). ERK1/2 inhibition with PD-98059 (10 µmol/L) abrogated increased contraction to phenylephrine in aorta (14±2 mN) and small mesenteric arteries (10±2 mN) from male DOCA rats, without any effects in arteries from male uninephrectomized or female animals. Compared with the other groups, phosphorylated ERK1/2 levels were increased in the aorta from male DOCA rats, whereas mitogen-activated protein kinase phosphatase 1 expression was decreased. Interleukin-10 plasma levels, which positively regulate mitogen-activated protein kinase phosphatase 1 activity, were reduced in male DOCA-salt rats. We speculate that augmented vascular reactivity in male hypertensive rats is mediated via activation of the ERK1/2 pathway. In addition, mitogen-activated protein kinase phosphatase 1 and interleukin 10 play regulatory roles in this process. (Hypertension. 2010;55:172-179.)

Key Words: ERK1/2 ■ MKP-1 ■ hypertension ■ sex differences ■ vascular reactivity

Hypertension, as well as other cardiovascular diseases, is more common in men than in women of similar age. Several studies on experimental models of hypertension, including mineralocorticoid hypertension, have shown that women do not develop elevated blood pressure as quickly or as severely as men. However, the mechanisms responsible for sex differences in salt-sensitive hypertension have not been completely elucidated. Involvement of increased vascular reactivity seems likely because contractile stimuli are increased in male desoxycorticosterone acetate (DOCA)-salt rats compared with females.

Extracellular signal–regulated kinase (ERK)1/2, a member of the mitogen-activated protein kinase (MAPK) family, has been reported to play a role in vascular dysfunction associated with mineralocorticoid hypertension. At the molecular level, ERK1/2 activation can be modulated by various mechanisms. Accordingly, MAPK phosphatase 1 (MKP-1; also known as dual-specificity phosphatase 1) plays an important role in dephosphorylation of ERK1/2 and deactivation of the ERK1/2 pathway. When MKP-1 is phosphorylated, its degradation is inhibited and, consequently, MKP-1 activation is increased.

Therefore, we hypothesized that, compared with female rats, ERK1/2 signaling is upregulated in the vasculature of male DOCA rats and contributes to sex-related differences in contractile responses in mineralocorticoid hypertension. We
also sought to determine whether decreased MKP-1 activity provides a mechanism leading to augmented ERK1/2 activation.

**Methods**

**Animals**

Male and female Sprague-Dawley rats (10 weeks old, 250 to 300 g; Harlan, Indianapolis, IN) were used in all of the studies. All of the procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education. The animals were housed on a 12-hour light/dark cycle and fed a standard chow diet with water or saline ad libitum.

**DOCA-Salt Hypertension**

Rats were unilaterally nephrectomized, and DOCA (200 mg per animal) pellets were implanted SC in the scapular region. DOCA rats received water containing 1% NaCl and 0.2% KCl for 3 weeks. Control rats (UNI) were unilaterally nephrectomized and received silastic pellets without DOCA and tap water.

**Blood Pressure Recordings by Telemetry and Tail Cuff Plethysmography**

Surgery was performed on UNI and experimental rats to implant blood pressure radiotelemetry transmitters (Data Sciences PA-C20, International), as described previously. Briefly, a midline incision was used to expose the abdominal aorta that was briefly occluded to allow for insertion of the transmitter catheter. The catheter was secured in place with tissue glue. The transmitter body was sutured to the abdominal wall along the incision line as the incision was closed. The skin was closed with staples that were removed 7 to 10 days after the incision was healed. Rats were allowed to recover from surgery and returned to individual housing for ≥1 week before data acquisition was initiated.

Vascular function and molecular experiments were conducted in separate experiments in groups of 6 rats. In this new set of experiments, systolic blood pressure was measured by tail-cuff plethysmography in conscious rats, 1 day before the end of the treatment, to assure that they developed hypertension and that the differences in blood pressure persisted until the end of the treatment. After 21 days, the rats were euthanized, blood was collected, and aorta and mesentery were isolated for further studies (see below).

**Vascular Functional Studies**

After euthanization, the mesentery and thoracic aortas were rapidly excised and placed in ice-cold physiological saline solution. Second-order branches of the mesenteric artery (~2 mm in length with an ID of ~100 to 200 μm) and thoracic aorta (4 mm in length) were carefully dissected. The second-order mesenteric arteries were mounted in tissue chambers for measurement of contractile force, as described previously. Both dissection and mounting of the vessels were carried out in cold (4°C) physiological saline solution. The segments were adjusted to maintain a passive force of 3 mN for the second-order mesenteric arteries and 30 mN for the aortic rings. Vessels were equilibrated for 60 minutes in physiological saline solution at 37°C and continuously bubbled with 5% CO₂ and 95% O₂. Arterial integrity was assessed first by stimulation of vessels with KCl (120 mmol/L) and, after washing and a new stabilization time, by contracting the segments with phenylephrine (PE; 1 μmol/L). Endothelium integrity was assessed by contracting the segments with PE, followed by stimulation with acetylcholine (10 μmol/L). Concentration-response curves to PE (1 nmol/L to 100 mol/L) were performed in the presence or absence of PD-98059 (10 μmol/L), an ERK1/2 inhibitor, in aorta and second-order mesenteric arteries. PD-98059 is a selective inhibitor of ERK1/2 activation that acts as an allosteric inhibitor, binding outside the ATP- and ERK1/2-binding sites on MAPK/ERK kinase 1/2. The modification of the 3D structure of MAPK/ERK kinase 1/2 renders it not phosphorylatable by upstream kinases.

**Western Blot Analysis**

Proteins (40 μg) extracted from aortas were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. None specific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween for 1 hour at 24°C. Membranes were then incubated with antibodies (1:1000) overnight at 4°C. Antibodies were as follows: p44/42 MAP kinase (ERK1/2), phospho-p44/42 MAP kinase (ERK1/2-Thr202/Tyr204), MKP-1, phospho--MKP-1 (Ser359), phospho-Elk-1 (Ser383), signal transducer and activator of transcription (Stat) 3, and phospho--Stat-3 (Ty705). MKP-1 was purchased from Abcam, and all of the others antibodies were from Cell Signaling Technology, Inc. Immunoblots for nonphosphoproteins were carried out in the same membranes used to evaluate their phosphorylated forms. After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. Results are normalized to β-actin protein and expressed as arbitrary units.

**Interleukin 10 Plasma Levels**

Plasma levels of interleukin 10 (IL-10) were determined by ELISA (Pierce Rat IL-10 Colorimetric ELISA kit), according to the manufacturer’s instructions.

**Drugs and Solutions**

Physiological saline solution of the following composition was used: 130.00 mmol/L of NaCl, 14.90 mmol/L of NaHCO₃, 4.70 mmol/L of KCl, 1.18 mmol/L of KH₂PO₄, 1.17 mmol/L of MgSO₄·H₂O, 5.50 mmol/L of glucose, 1.56 mmol/L of CaCI₂·2 H₂O, and 0.026 mmol/L of EDTA. PE hydrochloride and acetylcholine were purchased from Sigma Chemical Co. PD-98059 was purchased from Calbiochem. All of the reagents were of analytic grade. Stock solutions were prepared in deionized water or dimethyl sulfoxide (PD-98059). Control solutions containing vehicle levels of dimethyl sulfoxide were used through the experimental protocols.

**Data Analysis**

Results are presented as mean ± SEM. Contractions were recorded as changes in the displacement (meganewtons) from baseline and are represented as meganewtons for “n” experiments. Concentration-response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 4.0, GraphPad Software Inc). Statistically significant differences were calculated by ANOVA or by Student t test where appropriate. *P* < 0.05 was considered significant.

**Results**

**Sex Differences in Mean Arterial Pressure**

Twenty-four–hour mean arterial pressure (MAP), assessed by telemetry, was similar in male and female Sprague-Dawley rats before beginning DOCA-salt treatment and for the first 3 days of DOCA-salt treatment (Figure 1). From days 4 to 8, MAP increased 30 to 35 mm Hg in male DOCA-salt rats. In the same period, MAP augmented 20 to 25 mm Hg in female DOCA-salt rats, showing that sex-related differences in MAP occur in early stages of hypertension development. After day 10, blood pressure continued to increase in both groups, and differences were greater in male than in female DOCA-salt rats and persisted until the end of the treatment. At day 21, male DOCA rats displayed higher blood pressure (191 ± 3 mm Hg) compared with female DOCA rats (172 ± 7 mm Hg; Figure 1).

Difference in the pressoric levels at the end of the treatment was confirmed by tail-cuff plethysmography in a new set of
Sex Differences in PE-Induced Vasoconstriction

Concentration-response curves to PE, an α-1 adrenergic agonist, were performed to address sex-related differential regulation of vascular reactivity to contractile stimuli. Aorta from male DOCA-salt rats displayed increased vasoconstriction to PE compared with male UNI (Emax: 22.0±2.7 mN vehicle versus 16.9±2.4 mN, respectively; P<0.05; Figure 2A). Aortas from female DOCA-salt and female UNI rats displayed similar PE-induced contractile responses (Emax: 15.2±1.1 mN vehicle versus 16.9±2.4 mN, respectively; Figure 2B). From these results, we observed that PE-induced contraction is augmented in aortas from male but not female DOCA-salt rats.

It has been shown that the ERK1/2 pathway is upregulated in vessels from male DOCA-salt rats7,8,14 and that ERK1/2 increases force generation in arterial smooth muscle. We sought to investigate whether treatment with PD-98059, an ERK1/2 inhibitor, would interfere with the contractile responses to PE in vessels from male and female DOCA-salt hypertensive rats. Accordingly, ERK1/2 inhibition (PD-98059 to 10 μmol/L) reduced PE-induced contraction in aortas from male DOCA-salt rats (Emax: 2.7 mN vehicle versus 1.3 mN PD-98059; P<0.05; Figure 2A). After ERK1/2 inhibition, the sex difference in PE contraction in aortic rings from male and female DOCA-salt rats was abolished.

The effects of PD-98059 on −log EC50 (pD2) values for PE-induced contraction in aortas of male and female rats are summarized in the Table. Aortas from DOCA male and DOCA female rats displayed increased sensitivity to PE compared with their respective UNI counterparts, which was normalized after inhibition of ERK1/2. Sex differences in the sensitivity to PE-induced contraction were observed in the DOCA-salt group; male hypertensive rats displayed augmented sensitivity to PE compared with females.

![Figure 1. Male DOCA-salt rats display higher blood pressure compared with female DOCA-rats. Telemetry was used to assess blood pressure in male and female DOCA-salt rats. Data are mean±SEM (n=5). *P<0.05 vs female DOCA rats.](hypertension.ahajournals.org)
10.9 ± 0.8 mN) versus female UNI rats (E_{max}: 8.7 ± 1.7 mN; Figure 3B). Compared with female DOCA rats, PE-induced contraction was greater in small-mesenteric arteries from male DOCA rats. Blockade of ERK1/2 (PD-98059 to 10 μM/L) blunted PE-induced contraction in resistance-mesenteric arteries from male DOCA rats (E_{max}: 9.9 ± 0.9; P < 0.05; Figure 3A). However, no differences in PE-induced contractions were observed in resistance-mesenteric arteries from either male or female UNI rats or female DOCA rats (Figure 3). After ERK1/2 inhibition, differences in PE contraction in resistance-mesenteric arteries from male and female DOCA-salt rats were abolished.

The effects of PD-98059 on pD_{2} values for PE-induced contraction in small mesenteric arteries of male and female rats are summarized in the Table. Small mesenteric arteries from male and female DOCA rats displayed increased sensitivity to PE compared with their respective UNI counterparts. The inhibition of ERK1/2 decreased this sensitivity to PE in arteries from male DOCA rats but not in the females. No sex differences in the sensitivity to PE-induced contraction were observed among the groups after inhibition of ERK1/2.

**Sex Differences in ERK1/2 Activation**

Our next goal was to confirm augmented ERK1/2 activation with molecular analyses. When activated, ERK1/2 is phosphorylated at residues Thr202/Tyr204. Therefore, phosphorylation of Thr202/Tyr204 was used as a molecular parameter to measure ERK1/2 activation. No differences in total ERK1/2 protein levels were observed among the groups (Figure 4A and 4B). Phosphorylation of ERK1/2 at Thr202/Tyr204 was increased in aortas from male DOCA-salt rats in comparison with the others groups (Figure 4A and 4C). In agreement with the functional data, these results support the hypothesis that ERK1/2 is more activated in arteries from male DOCA rats compared with female rats.

ERK1/2 can be modulated by several mechanisms at almost every step of the pathway. Therefore, further studies were performed to determine upstream alterations on the ERK1/2 pathway. No differences in phosphoinositide-3 kinase, c-Raf, or ERK kinase (MAPK/ERK kinase 1/2) expressions, for total or phosphorylated forms, were found between aortas from male or female DOCA rats (data not shown). Investigating downstream proteins from the ERK1/2, we found that total and phosphorylated Stat-3 (Figure 5A through 5C) and ELK-1 (Figure 5A and 5D) were augmented in aortas from male DOCA-salt rats compared with aortas from the other groups.

Proteins in the MAPK pathway are activated through phosphorylation, whereas dephosphorylation of kinases, which is mediated by phosphatases, leads to inactivation. Therefore, we determined whether differential expression of the MKP-1, a phosphatase that regulates ERK1/2 activation, occurs in aortas from male and female DOCA-salt rats. Phosphorylation of MKP-1 (Ser359), but not total MKP-1, was significantly reduced in aortas from male DOCA-salt rats, and a small, but not significant, reduction was observed in aortas from female DOCA-salt rats (Figure 6A through 6C). Considering that MKP-1 phosphorylation at Ser359 is a
modulator site for MKP-1 activation, we observed a decreased activity of MKP-1 in aortas from DOCA-salt rats (Figure 6D), and we speculate that this is an important modulator of sex-related differential vascular activation of the ERK1/2 pathway in DOCA-salt hypertension.

**IL-10 Plasma Levels and Sex Differences During DOCA-Salt Hypertension**

MKP-1 activity is largely modulated by several proinflammatory and anti-inflammatory stimuli, including IL-10, an anti-inflammatory cytokine. Cytokines, such as IL-10, positively regulate MKP-1. Moreover, IL-10 has a protective role in conditions where activation of ERK1/2 is increased. We found that IL-10 plasma levels were decreased in male DOCA-salt rats, and a small, but not significant, reduction was observed in female DOCA-salt rats (Figure 7), which suggests that IL-10 may contribute to sex differences in the activation of the ERK1/2 pathway, as well as in differential vascular reactivity in DOCA-salt hypertension.

**Discussion**

Male DOCA rats displayed more severe hypertension than females, and, consequently, they have increased risk for developing cardiovascular events and organ damage, which may shorten their life span. Our study shows that a short-term exposition to a severe increase in pressoric levels is able to impair vascular function to contractile stimuli, especially in male hypertensive rats. Therefore, the use of therapies to prevent or retard the development of vascular lesions would help patients to extend their life span.

Our study used a novel approach to address the well-known sex differences in DOCA-salt hypertension by investigating whether differential intracellular signaling, specifically, ERK1/2 activation, contributes to the sex-related differences observed in the vascular function of DOCA-salt hypertensive rats. Our functional and molecular data clearly show that the ERK1/2 pathway is overactivated in the vasculature of male DOCA-salt rats compared with UNI males, and this same increase in activity is not apparent with DOCA treatment in the female rats. Accordingly, increased activation of downstream proteins that are regulated by ERK1/2 phosphorylation, such as Stat-3 and ELK-1, was observed in aortas from male DOCA-salt rats. D’Angelo and Adam first showed that inhibition of ERK attenuates force development in the porcine carotid artery by lowering myosin light chain phosphorylation. In addition, it is known that ERK1/2 phosphorylates caldesmon and calponin, blocking their ability to inhibit actin and myosin interaction, leading to augmented contractions.

From our results, it is reasonable to speculate that the increased activation of the ERK1/2 pathway leads to the augmented vascular contractile responses seen in aortas from male DOCA-salt rats.

We then further probed the mechanisms leading to differential vascular activation of ERK1/2 phosphorylation in male and female UNI and DOCA rats. Activation of the MAPK pathway can be modulated at various steps, including recep-
tor desensitization, dissociation of signaling complexes from the receptor, and deactivation of pathway mediators. Our data reveal that proteins upstream of ERK1/2, such as phosphoinositide-3 kinase, c-Raf, and MAPK/ERK kinase 1/2, are not differentially activated in the vasculature of male and female DOCA-salt rats. Rather, our data suggest alterations in downstream proteins that regulate this process. Accordingly, phosphatases are important regulators of the MAPK pathway and are likely to be one of the most energy-efficient modes for deactivation of MAPK. MKP-1 belongs to a family of inducible nuclear dual-specificity phosphatases exerting catalytic activity to phosphotyrosine- and phosphothreonine-containing proteins. MKP-1 is known to inactivate ERK1/2, c-Jun N-terminal kinase, and p38 MAPK in vitro and in vivo, and both its transcription and activity are tightly regulated. MKP-1 is transcriptionally induced through ERK1/225 but also by other proteins, such as p53 and Jak2. Phosphorylation of MKP-1 at Ser359 and Ser364, on its carboxy-terminal region, inhibits MKP-1 degradation, contributing to increased activity of this phosphatase. Here we observed that the phosphorylation of MKP-1 was decreased in aortas from male DOCA-rats compared with UNI males and females, suggesting a smaller inhibitory effect of this phosphatase on the ERK1/2 pathway. Several phosphatases, including MKP-1, are posttranslationally regulated through phosphorylation. Although phosphorylation is not needed for activation of the phosphatases, it does alter their stability. It was shown recently that, under inflammatory conditions, IL-10 prolongs the expression of MKP-1 of 2- to 3-fold. This accumulation of MKP-1 would lead to greater MKP-1 activity or vice versa.

One question that remains is how MKP-1 is being differently modulated between male and female DOCA-salt rats. It is well known that markers of inflammation have been shown to be upregulated in different forms of cardiovascular disease and to correlate with vascular risk. In addition, vascular dysfunction in DOCA-salt hypertension has been extensively studied, and a positive correlation with increased inflammatory mediators has been reported. Moreover, cytokines play an important role regulating MKP-1 activity. It was shown recently that, under inflammatory conditions, IL-10 prolongs the expression of MKP-1, accelerating the inactivation of MAPK without diminishing peak activity.
tion, it has been suggested that MKP-1 mediates anti-inflammatory effects of IL-10.\textsuperscript{16} The deletion of MKP-1 resulting in a substantial increase in the production of IL-10 is a probable tentative marker in the upregulation of MKP-1.\textsuperscript{31,32}

Our results show that IL-10 plasma levels are reduced in male DOCA-salt rats. Therefore, we speculate that a positive correlation between lower levels of IL-10 and decreased phosphorylation of MKP-1 contributes to sex-related differences in ERK1/2 activation and vascular dysfunction in DOCA-salt hypertension. We have demonstrated recently that IL-10 plays a protective role in the vasculature, via inhibitory effects on ERK1/2 activity, in mice that were chronically infused with tumor necrosis factor-\textalpha.\textsuperscript{12} Accordingly, others have reported that IL-10 plays a protective role in the alterations of vascular reactivity.\textsuperscript{33–35} We cannot rule out that other pathways can contribute to decreased MKP-1 activity. Therefore, other pathways, such as those activated by the endothelin 1 system, should be further evaluated in future studies.

Reactive oxygen species, such as superoxide anion, are increased in the vasculature of DOCA-salt rats.\textsuperscript{36,37} In addition, it has been shown that ERK1/2 activation, via superoxide anion, contributes to spontaneous contractile tone in isolated rat aortas,\textsuperscript{38} indicating that ERK1/2 activation and superoxide anion are closely related. Therefore, oxidative stress can be one additional mechanism that contributes to the augmented vascular contraction.

It remains to be understood which mechanisms are contributing to the development of DOCA-salt hypertension in female rats. Accordingly, it seems that estrogen has an important functional influence in the pressure level, but other mechanisms, such as oxidative stress and inflammation, can also contribute.\textsuperscript{39–41}

In conclusion, DOCA-salt hypertension in males is associated with increased vascular contraction via upregulation of the ERK1/2 pathway because of downregulation of MKP-1. In addition, IL-10 seems to play a positive regulatory role on MKP-1, and this mechanism is decreased in male DOCA-salt rats.

**Perspectives**

This is the first study showing that ERK1/2-related mechanism, which contributes to vascular contraction, is impaired in male DOCA-salt rats when compared with female hypertensive rats. In addition, MKP-1, a phosphatase that prevents ERK1/2 activation, is preserved in arteries from DOCA-salt female rats, contributing to the vascular protection observed in females. A major challenging in this field will be to better understand mechanisms that contribute to the regulation of the ERK1/2 pathway activation and how sex-differences are being modulated.

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**Disclosures**

None.


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