Angiotensin Type 1a Receptors in the Subfornical Organ Are Required for Deoxycorticosterone Acetate-Salt Hypertension

Aline M. Hilzendeger, Martin D. Cassell, Deborah R. Davis, Harald M. Stauss, Allyn L. Mark, Justin L. Grobe, Curt D. Sigmund

Abstract—Although elevated renin–angiotensin system activity and angiotensinergic signaling within the brain are required for hypertension, polydipsia, and increased metabolic rate induced by deoxycorticosterone acetate (DOCA)-salt, the contribution of specific receptor subtypes and brain nuclei mediating these responses remains poorly defined. We hypothesized that angiotensin type 1a receptors (AT₁aR) within the subfornical organ (SFO) mediate these responses. Transgenic mice carrying a conditional allele of the endogenous AT₁R (AT₁R<sub>Cre<sub>lox</sub></sub>) were administered an adenovirus encoding Cre-recombinase and enhanced green fluorescent protein (eGFP) or adenovirus encoding eGFP alone into the lateral cerebral ventricle. Adenovirus encoding Cre-recombinase reduced AT₁R mRNA and induced recombination in AT₁R<sub>Cre<sub>lox</sub></sub> genomic DNA specifically in the SFO, without significant effect in the paraventricular or arcuate nuclei, and also induced SFO-specific recombination in ROSA<sub>ΔTomato</sub> reporter mice. The effect of SFO-targeted ablation of endogenous AT₁R was evaluated in AT₁R<sub>Cre<sub>lox</sub></sub> mice at 3 time points: (1) baseline, (2) 1 week after virus injection but before DOCA-salt, and (3) after 3 weeks of DOCA-salt. DOCA-salt–treated mice with deletion of AT₁R in SFO exhibited a blunted increase in arterial pressure. Increased sympathetic cardiac modulation and urine copeptin, a marker of vasopressin release, were both significantly reduced in DOCA-salt mice when AT₁R was deleted in the SFO. Additionally, deletion of AT₁R in the SFO significantly attenuated the polydipsia, polyuria, and sodium intake in response to DOCA-salt. Together, these data highlight the contribution of AT₁R in the SFO to arterial pressure regulation potentially through changes on sympathetic cardiac modulation, vasopressin release, and hydromineral balance in the DOCA-salt model of hypertension. (Hypertension. 2013;61:716-722.) ● Online Data Supplement

Key Words: circumventricular organs ■ fluid balance ■ hypertension ■ mineralocorticoid ■ salt ■ site-specific receptor deletion

Approximately 25% of hypertensive patients exhibit low-renin hypertension that is associated with salt-sensitivity.<sup>1</sup> In animals, low-renin hypertension can be experimentally modeled with deoxycorticosterone acetate (DOCA)-salt treatment. Early studies showed that daily subcutaneous injections of DOCA with 1% NaCl drinking solution caused an elevation of arterial pressure in rats.<sup>2</sup> Importantly, the peripheral renin–angiotensin system (RAS), a major regulator of arterial pressure, is suppressed after chronic DOCA-salt treatment,<sup>3</sup> but despite this, the brain RAS is elevated.<sup>4,5</sup> Hyperactivity of the brain RAS has been implicated in the development and maintenance of hypertension in several experimental and genetic animal models,<sup>6</sup> and can also affect fluid balance and metabolism.<sup>7</sup> Angiotensin (Ang) II, one of the major bioactive peptides of the brain RAS, mediates most of its known functions through the Ang II type 1 receptors (AT₁R). AT₁R in mice.<sup>7</sup> However, the exact mechanisms and specific brain nuclei involved in mediating the effects of elevated brain RAS activity remain poorly understood.

The circumventricular organs, including the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and the area postrema are highly vascularized brain regions that respond to circulating signals. The circumventricular organs lack a blood–brain barrier and, thus, are key points of intercommunication between the blood, the brain parenchyma, and the cerebrospinal fluid. Numerous studies support their role in sodium and water balance, cardiovascular regulation, and energy metabolism.<sup>8</sup> The SFO has a number of axonal projections to cardiovascular control regions in the hypothalamus,<sup>8</sup> and some of these projections may use Ang-II as a neurotransmitter.<sup>9</sup> Lesions of the anteroventral third ventricle region, which include the SFO, abolish the effects of DOCA-salt on arterial pressure<sup>10</sup> and fluid intake,<sup>12</sup> suggesting that the SFO is likely involved in arterial pressure...
and fluid volume control. Previous studies have shown that central production and action of Ang-II through AT\textsubscript{1}R are crucial for DOCA-salt–induced hypertension.\textsuperscript{4,13} DOCA-salt increases the expression of Ang receptors in the SFO,\textsuperscript{14} and microinjection of Ang-II into the SFO induces drinking\textsuperscript{15} and pressor responses.\textsuperscript{16} Recent studies suggest there is an interaction between Ang-II and aldosterone in the brain, and that both AT\textsubscript{1}R and mineralocorticoid receptor are necessary for the hypertension induced by either.\textsuperscript{17} Here, we tested the hypothesis that specifically targeting the murine AT\textsubscript{1}aR in the SFO would impact arterial pressure and fluid balance in the DOCA-salt model.

Methods

Please see the online-only Data Supplement for a more detailed Methods section.

Animals

Male mice (12–18 weeks) containing a conditional allele of the endogenous AT\textsubscript{1}R (AT\textsubscript{1}R\textsuperscript{flox}) gene were studied (the number is indicated in the figures/legends).\textsuperscript{18} ROSA\textsuperscript{tdTomato} mice were obtained from the Jackson Laboratory (Stock 007914). Mice were maintained on standard laboratory chow and tap water ad libitum. All studies were approved by the University of Iowa Animal Care and Use Committee and was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery and DOCA-Salt Model

All measurements were performed in mice during baseline (before any treatment), 1 week after virus injection, and again 3 weeks after DOCA implantation (Figure 1A). Three separate cohorts of mice were used for arterial pressure, intake behavior, and indirect calorimetry. Recombinant adenovirus encoding Cre-recombinase and enhanced green fluorescent protein (eGFP) (AdGFP) alone, or AdGFP containing Cre-recombinase (AdCRE) obtained from the University of Iowa Vector Core was microinjected (1 µL, 10\textsuperscript{10} plaque-forming units/mL) into the lateral cerebral ventricle (ICV).\textsuperscript{19} After 1 week, the same cohort of mice underwent a subcutaneous implantation of a 50-mg pellet of DOCA (Sigma) performed under isoflurane anesthesia without uninephrectomy.\textsuperscript{20} Mice were singly housed after recovery and maintained on standard chow with ad libitum access to tap water and 0.15 mol/L NaCl for 4 weeks. Arterial pressure was measured by radiotelemetry (TA11PA-C10, Data Sciences International).\textsuperscript{5,20} Spectral analysis of heart rate (HR) was performed to assess autonomic modulation of cardiac function, and baroreflex sensitivity was determined using the sequence technique as detailed in the online-only Data Supplement.\textsuperscript{21} Resting metabolic rate was assessed by indirect calorimetry.\textsuperscript{5} Photomicrographs of the ROSA\textsuperscript{tdTomato} brains injected with AdCRE or AdGFP (ICV) were obtained from 16 brains (total 4 AdGFP and 12 AdCRE) using a Nikon fluorescence microscope, and SFO images were merged using Adobe Photoshop CS (version 8.0).

Statistics

Data were analyzed by 2-way ANOVA, with repeated measures as appropriate. Bonferroni post hoc tests were used to dissect treatment effects. Data sets failing normality or equal variance tests were subjected to nonparametric analyses, including Friedman ANOVA and Mann-Whitney U tests. Fold changes (mRNA) from the reverse transcriptase-polymerase chain reaction assays were analyzed using the Livak method.\textsuperscript{22} Differences were considered significant at \(P<0.05\). All data are presented as mean±SEM.

Results

AT\textsubscript{1}R\textsuperscript{flox} mice underwent the experimental protocol shown in Figure 1A. Experimental end points were measured at baseline, 7 days after AdCRE or AdGFP was administered, and during the third week after DOCA-salt.

Validation of SFO-Targeted AT\textsubscript{1}aR

Brains were collected from each mouse at the end of the protocol and microchips of SFO, paraventricular nucleus (PVN), arcuate nucleus (ARC), and cortex were used to evaluate the deletion of AT\textsubscript{1}aR. Expression of AT\textsubscript{1}aR mRNA was significantly decreased in the SFO of AdCRE–treated mice (Figure 2A). Decreased AT\textsubscript{1}aR mRNA expression was observed in PVN, but it was not statistically significant (Figure 2B). There was no change in the AT\textsubscript{1}aR mRNA expression in ARC (Figure 2C) or cortex (data not shown). We further evaluated the site-specificity of the AT\textsubscript{1}aR deletion by assaying for Cre-mediated recombination in genomic DNA. A polymerase chain reaction product representing the intact gene was detected in the SFO of mice treated with AdGFP, whereas in the AdCRE–treated group, the recombined allele was detected in 6 of 7 samples (Figure 2D). No recombination was observed in the cortex. Weak recombination was noted in the ARC, but little was observed in the PVN (Figure S1 in the online-only Data Supplement).

Rosa\textsuperscript{tdTomato} reporter mice were injected with AdCRE, and brains were collected 1 to 4 weeks after injection to further validate specificity. In this model, red fluorescence becomes apparent only after Cre-mediated recombination. Abundant

Figure 1. Experimental design. A, Schematic of the protocol used in the study. B, Schematic map of the intact and recombined angiotensin type 1a receptors (AT\textsubscript{1a}R\textsuperscript{flox}) allele. Primers for the genomic DNA polymerase chain reaction reaction are represented. DOCA indicates deoxycorticosterone acetate; and ICV, lateral cerebral ventricle.
TdTomato staining is evident that overlaps with eGFP (also encoded on AdCRE) and the 4'-6-Diamidino-2-phenylindole stain for nuclei (Figure 3A). Three other representative images of the SFO from different mice indicate robust red fluorescence in neuronal cell bodies and processes (Figure 3B). We also examined the OVLT, median preoptic nucleus (MnPO), supraoptic nucleus (SON), ARC, area postrema, and the PVN weekly for 4 weeks. There was no red fluorescence in any region in mice injected with AdGFP. One week after AdCRE, red fluorescence was detected in a few ependymal cells of the OVLT, MnPO, SON, ARC, area postrema, and PVN (Figure S2). This may account for the recombination noted at the genomic DNA level in the ARC. By 2 weeks, there was no signal in the OVLT, and by 3 weeks, the ependymal signal in the PVN was gone (Figure S3). However, there was a preservation of a robust red fluorescent signal in the SFO even 4 weeks after AdCRE. Together, our data measuring AT$_{1}$R mRNA, recombination of AT$_{1}$R$^{lox}$ genomic DNA, and the ROSA$^{tdTomato}$ reporter mouse indicate that our ICV injection is efficient at specifically targeting the SFO.

**SFO AT$_{1}$R Are Necessary for DOCA-Salt Hypertension**

Arterial pressure was unchanged at baseline in groups destined to receive either AdCRE or AdGFP, and virus administration did not change arterial pressure (Figure 4A). DOCA-salt treatment increased arterial pressure by $\approx 30$ mm Hg in the AdGFP mice (Figure 4B), whereas deletion of AT$_{1}$R in the SFO blunted the increase in arterial pressure by DOCA-salt (Figure 4A and 4B). Interestingly, HR decreased to a similar degree in both AdGFP and AdCRE mice in response to DOCA-salt (Figure 4C).

Sympathetic activation and impaired baroreflex function are closely related to increased BP in the DOCA-salt model of hypertension. Therefore, we studied autonomic modulation of cardiac function using spectral analysis of HR and baroreflex function using the spontaneous sequence technique. Interestingly, the increases in low frequency HR variability (which reflects both sympathetic and parasympathetic modulation) seen with DOCA-salt were blunted by AdCRE (Figure 4D). High-frequency HR variability (which reflects only parasympathetic cardiac modulation) did not differ between the 2 groups (Figure 4E), suggesting that the blunted low frequency HR variability in the AdCRE-treated mice reflects reduced sympathetic cardiac modulation. Baroreceptor-HR reflex sensitivity tended to be larger in the AdCRE group compared with the AdGFP control group after DOCA-salt (Figure S4).

Increased arginine vasopressin (AVP) release has been reported to be required for DOCA-salt hypertension. Consequently, we estimated AVP release by measuring urine copeptin, a stable product from the preproAVP produced in a 1:1 ratio with AVP. Like arterial pressure, urine copeptin was equivalent at baseline and after virus, and the increase caused by DOCA-salt was significantly blunted by AdCRE (Figure 4F).

**SFO AT$_{1}$R Are Necessary for DOCA-Salt–Induced Changes in Fluid Balance**

Fluid intake was equal at baseline and after virus, but markedly increased in response to DOCA-salt (Figure 5A). A marked
increase in urine output (Figure 5B), sodium intake (Figure 5C), and sodium excretion (Figure 5D) was also observed after DOCA-salt. The induction of each of these phenotypes was blunted in the AdCRE-treated mice. Male DOCA-salt mice exhibited a mild hypernatremia that was blunted by AdCRE (Table). Hypokalemia was not altered by AdCRE.

**SFO AT1aR Are Not Required for DOCA-Salt–Induced Changes in Metabolic Rate**

Body weight was unchanged, whereas blood glucose was decreased by DOCA-salt but was unaffected by deletion of SFO AT1aR (Table). Oxygen consumption during the baseline and virus time periods was not different between groups (Figure 6). DOCA-salt increased oxygen consumption, but this increase was not affected by the deletion of AT1aR in the SFO.

**Discussion**

The main findings from these studies are that AT1aR in the SFO, and possibly other nuclei, such as the PVN, contribute to the effects of DOCA-salt on arterial pressure and fluid and sodium intake, but are not required to mediate the DOCA-salt–induced increase in oxygen consumption. These data support the hypothesis that AT1aR signaling in the SFO is important for DOCA-salt hypertension, and further suggest that sympathetic activation and vasopressin release are possible mechanisms.

Human hypertensive patients more frequently exhibit depressed than elevated circulating RAS activity.1 Despite this, very few models of low-renin hypertension are commonly studied. DOCA-salt treatment, first reported to have hypertensive effects in rodents,2 is a model of low-circulating–RAS activity that correlates with (and is dependent on) elevated brain RAS activity.4,13 Previous pharmacological studies have demonstrated that RAS peptide production and action in or near the cerebral ventricles and within the hypothalamus are required for DOCA-salt hypertension.4,13 Similarly, early work supported the concept of cross-talk between mineralocorticoids and the RAS within the brain in the control of hypertension.

**Figure 4.** Effects of angiotensin type 1a receptors (AT1aR) deletion in the subfornical organ (SFO) on deoxycorticosterone acetate (DOCA)-salt hypertension. A–C, Twenty-four hour mean arterial pressure (MAP, A), changes in MAP compared with baseline (B), and heart rate (C). D–D, Spectral analysis of heart rate (HR) in low-frequency (sympathetic and parasympathetic cardiac modulation) and (E) high frequency (parasympathetic cardiac modulation). F, Measurements of urine copeptin. The number is indicated in each bar. *P<0.05 vs adrenovirus encoding eGFP alone (AdGFP); †P<0.05 vs virus. All data are mean±SEM.

**Table.** Body Weight and Blood Analyses in Nontreated Mice (Control) and DOCA-Salt–Treated AdGFP and AdCRE

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Blood Na, mmol/L</th>
<th>Blood K, mmol/L</th>
<th>Blood Glucose, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.8±0.5</td>
<td>148±0.5</td>
<td>6.9±0.2</td>
<td>204±10</td>
</tr>
<tr>
<td>AdGFP</td>
<td>28.3±0.6</td>
<td>155±1.2</td>
<td>4.3±0.3</td>
<td>172±8</td>
</tr>
<tr>
<td>AdCRE</td>
<td>29.7±0.6</td>
<td>149±2.8</td>
<td>4.7±0.6</td>
<td>166±8</td>
</tr>
</tbody>
</table>

AdCRE indicates adenovirus encoding Cre-recombinase; AdGFP, adenovirus encoding eGFP alone; and DOCA, deoxycorticosterone acetate.

*P<0.05 vs control.
and AT1aR gene ablation occurred in regions that we did not cannot completely rule out the possibility that recombination  

current study, DOCA-salt also increases aldosterone levels and central expression of mineralocorticoid receptors, and an interaction between the actions of Ang-II and aldosterone in the brain in hypertension has been reported. Future studies will be necessary to unravel the importance of this cross-talk in the SFO.

It is notable that SFO-targeted ablation of AT 1aR had a minimal impact on baseline arterial pressure or fluid intake, whereas it effectively attenuated the fluid turnover and arterial pressure effects of DOCA-salt. Our data and other studies suggest that AT 1aR in the SFO may be dispensable under normal conditions, but are essential to mediate arterial pressure and fluid intake responses after Ang-II and DOCA-salt. Furthermore, roles for both vasopressin and the sympathetic nervous system in DOCA-salt hypertension have been previously established, and our data are consistent with contributions of the SFO in the control of both of these mechanisms. It is notable that lesioning the SFO significantly reduces vasopressin secretion after central Ang-II. Whereas the PVN and SON are known to produce AVP, the SFO does not. Our data suggest that AVP release in DOCA-salt is associated with AT 1R signaling in the SFO. Consequently, the deletion of AT 1R in the SFO could influence AVP production in these nuclei by virtue of the efferent projections from the SFO to the PVN and SON, some of which may be angiotensinergic.

Although the importance of the SFO in regulating fluid intake is undisputed, its role in regulating arterial pressure is less clear, as in rats lesions of the SFO attenuate Ang-II–induced hypertension, but do not attenuate DOCA-salt–induced hypertension. On the contrary, lesions of the anteroventral third ventricle, which may ablate efferent projects from the SFO to sites in the anteroventral third ventricle region, prevent DOCA-salt–induced hypertension. Studies in mice, where angiotensinogen expression is specifically ablated in the SFO, support the importance of this region in mediating responses to Ang-II. It remains unclear whether the finding here of a role for the SFO in mediating arterial pressure responses to DOCA-salt is attributable to species-specific differences (lesion studies in rats versus genetic studies in mice), or are related to the different methodologies used. Physical lesions of the SFO cause a complete loss of neuronal connectivity, a loss of both excitatory and inhibitory outputs. This could cause widespread changes in functional activity in areas targeted by the SFO and release compensatory mechanisms that may mask the role of the SFO. In contrast, highly specific deletion of a single receptor, in this case the AT 1R, may affect only a single SFO output that is critical to arterial pressure regulation, a notion consistent with evidence for multiple brain RAS mechanisms.

We previously demonstrated that DOCA-salt treatment of wild-type mice results in elevated resting metabolic rate, and that ICV infusion of the AT 1R antagonist losartan significantly
attenuated this effect.20 Interestingly, lesion studies suggest that the SFO may mediate some thermogenic effects of Ang-II in rats.34 Moreover, sRA double transgenic mice, which exhibit abundant Ang-II in the SFO, exhibit increased metabolic rate.3 It was, therefore, surprising that SFO-targeted deletion of the AT1aR did not attenuate the induction of resting metabolism by DOCA-salt. These findings may be explained by the metabolic protective effects of ICV losartan being mediated by a different receptor subtype (eg, AT1bR), that the bio-distribution of losartan and AdCRE administered ICV may be different, and that AT1R receptors within the SFO are not involved in the control of resting metabolic rate. The latter would suggest that AT1R in other nuclei, which can be accessed by ICV losartan, may play a role in regulating the metabolic responses to DOCA-salt. AT1R are present at high density in the MnPO, an area often associated with osmo- and thermoregulation,35 and AT1R-containing and heat-sensitive thermoregulatory neurons are localized in the MnPO.36 Angiotensinergic projections between the SFO and MnPO have been electrophysiologically and functionally characterized.12 Similarly, AT1R have also been identified in the ARC, a site most often identified in the regulation of food intake, and the classical site of leptin action.37 Ang-II–induced neuronal activity in the PVN can be augmented by changes in temperature, suggesting that the PVN may be thermoregulatory in response to Ang-II.36 Indeed, the SFO has angiotensinergic projections to the PVN, and Ang-II causes neuronal activation of PVN neurons.10 Further studies will be needed to assess whether any of these regions, perhaps in cooperation with AT1R in the SFO, play a role in mediating the metabolic effects of DOCA-salt.

Perspectives

Our recent studies in DOCA-salt mice and previously in double transgenic sRA, both models of hyperactivity of brain RAS, advance the concept that the brain RAS plays an important role in BP control, fluid turnover, and energy expenditure. In the present study, we have reported that AT1R signaling in the SFO is important for DOCA-salt–induced hypertension and influences sympathetic cardiac modulation, increases vasopressin release, and robustly stimulates fluid intake. On the contrary, ablating AT1R signaling within the SFO is alone insufficient to attenuate increased resting metabolic rate in the DOCA-salt model. We have previously provided compelling evidence supporting the hypothesis that there are differential efferent mechanisms regulating arterial blood pressure, fluid turnover, and metabolic rate by the brain RAS.5 These new data suggest divergent region-specific signaling, some angiotensinergic, may also be mediating these responses.

Acknowledgments

We acknowledge the intellectual assistance of Dr Pimonrat Ketwatsamkron and Dr Henry L. Keen. We thank Norma Sinclair, Patricia Yarolem, and JoAnn Schwartz from the University of Iowa Transgenic Animal Facility for genotyping mice.

Sources of Funding

This work was supported by the National Institutes of Health (NIH) Program Project Grant HL084207 (A.M. Hilzendeger and C.D. Sigmund), HL058048, and HL061446 (C.D. Sigmund), an NIH Pathway to Independence K99/R00 award HL098276 (J.L. Grobe), by endowed chairs and funds from the Roy J. and Lucille A. Carver Trust (A.L. Mark and C.D. Sigmund), and by an American Heart Association Midwest Affiliate Postdoctoral Fellowship 11POST3610024 (A.M. Hilzendeger).

Disclosures

None.

References


---

**Novelty and Significance**

**What Is New?**

- Angiotensin type 1a receptors (AT\(_1\)R) in the subfornical organ (SFO) are important for mediating increases in arterial pressure in deoxycorticosterone acetate (DOCA)-salt mice.

- DOCA-salt-induced changes on vasopressin release, sympathetic cardiac modulation, and fluid turnover were attenuated when AT\(_1\)R were specifically deleted in the SFO.

- Ablation of angiotensin signaling through AT\(_1\)R within the SFO did not affect DOCA-salt–induced increases in resting metabolic rate.

**What Is Relevant?**

- The SFO is a circumventricular organ accessible to many circulating endogenous factors and pharmacological compounds, and AT\(_1\)R in this important area of the brain are required to fully mediate the arterial pressure increases in the DOCA-salt model of experimental hypertension.

**Summary**

These experimental findings suggest that AT\(_1\)R in the SFO are necessary to mediate the blood pressure and fluid intake responses in DOCA-salt hypertension. The reduction in DOCA-salt hypertension after SFO-targeted ablation of AT\(_1\)R may be attributable to blunted increases in AVP release and fluid intake, or changes in sympathetic cardiac modulation. We previously showed that blockade of central AT\(_1\)R by lateral cerebral ventricle Losartan can blunt the increase in resting metabolic induced by DOCA-salt. That SFO-targeted ablation of AT\(_1\)R did not alter resting metabolic rate implies a divergence in the AT\(_1\)R-dependent mechanisms and AT\(_1\)R-containing sites in the brain which control the cardiovascular and metabolic phenotypes induced by DOCA-salt. Consequently, our study has broad implications in cardiovascular and metabolic control, which could provide new insights into fundamental mechanisms in hypertension.
Angiotensin Type 1a Receptors in the Subfornical Organ Are Required for Deoxycorticosterone Acetate-Salt Hypertension
Aline M. Hilzendeger, Martin D. Cassell, Deborah R. Davis, Harald M. Stauss, Allyn L. Mark, Justin L. Grobe and Curt D. Sigmund

Hypertension. 2013;61:716-722; originally published online December 24, 2012; doi: 10.1161/HYPERTENSIONAHA.111.00356
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/61/3/716

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2012/12/24/HYPERTENSIONAHA.111.00356.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENTAL MATERIAL

CHBPR:
Angiotensin AT_{1A} Receptors in the Subfornical Organ are Required for DOCA-Salt Hypertension

Aline M. Hilzendeger¹, Martin D. Cassell², Deborah R. Davis³, Harald M. Stauss⁴, Allyn L. Mark¹, Justin L. Grobe³ and Curt D. Sigmund³

Departments of Internal Medicine¹, Anatomy & Cell Biology² and Pharmacology³, Roy J. and Lucille A. Carver College of Medicine
Department of Health & Human Physiology⁴, College of Liberal Arts & Sciences
University of Iowa, Iowa City, IA
DETAILED METHODS

Animals: Mice containing a conditional allele of the endogenous AT₁aR (AT₁aR<sup>flox</sup>) were a generous gift from Lisa A. Cassis and Alan Daugherty (University of Kentucky) and maintained on a C57BL/6 background.<sup>1</sup> The reporter mouse ROSA<sup>TdTomato</sup> was obtained from the Jackson Laboratory (Stock 007914) and male mice aged 12-16 weeks were used to confirm the specificity of AdCRE. Animal care rooms were maintained on a 12:12 light/dark cycle, between 21°C and 23°C, and 15% to 40% relative humidity. Standard laboratory chow (Harlan Teklad, NIH-31 modified 6% mouse/rat diet) and tap water were provided ad libitum. All mouse work was approved by the University of Iowa Animal Care and Use Committee and was performed in accordance with the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Brain surgery and DOCA-salt model: For adenovirus injections, mice were anesthetized by inhalation of isoflurane and the head was placed in a stereotaxic frame (David Kopf Instruments). Under aseptic conditions, the skin was incised and an opening made in the skull. Recombinant adenovirus encoding Cre-recombinase and eGFP (AdCRE) or eGFP alone (AdGFP) obtained from the Iowa Gene Transfer Vector Core was microinjected (volume of 1µL, 10<sup>10</sup> plaque-forming units/mL) into the lateral cerebral ventricle (1.0 mm lateral, 0.3 mm posterior, 3.0 mm ventral to bregma). After one week of measurements, the same cohort of mice underwent a subcutaneous implantation of a 50-mg pellet of DOCA (Sigma) performed under isoflurane anesthesia. Animals were not uninephrectomized as we previously reported.<sup>2</sup> Mice were singly housed after recovery and were maintained on standard chow and had ad libitum access to both tap water and a 0.15 mol/L (0.9%) NaCl drinking solution for 4 weeks. Measurements were performed after 3 weeks of DOCA-salt treatment. Three different cohorts of mice were utilized for arterial pressure, intake behavior and indirect calorimetry.

Arterial Pressure: Arterial pressure was measured by radiotelemetry (TA11PA-C10, Data Sciences International) as previously described implanting the catheter into the left carotid artery.<sup>2,3</sup> After one week of recovery arterial pressure was recorded during the baseline period. The same cohort of mice underwent ICV microinjection of virus (AdCRE, N=11 or AdGFP, N=10), and were allowed to recover for one week before arterial pressure measurements were obtained during the following week. The third period of measurement was obtained in the same cohort of mice that underwent a DOCA pellet implantation subcutaneously in the space between the scapulae. Due to consequences of the surgeries and length of the protocol, a number of mice died before the last measurement of arterial pressure (DOCA-salt). Mice that survived (AdCRE, N=5 or AdGFP, N=8, as represented in the bar graphs) were healthy and presented a good telemetry signal. Arterial pressure was recorded during the third week of DOCA-salt treatment. All recordings were collected for 10 seconds every 5 minutes for 5 consecutive days and analyzed with Dataquest software (DSI). Data from each animal was averaged hourly, and corresponding times across the 5 days were averaged for each animal to create a single composite 24-hour tracing. During one to two hours of measurement during each treatment, blood pressure of each mouse was recorded continuously using a higher sampling rate of 2000Hz for frequency domain analysis and for baroreceptor reflex testing using the sequence technique. Spectral analysis of heart rate (HR) was performed to assess autonomic modulation of cardiac function. Briefly, beat-by-beat HR time series were derived from the BP waveforms and converted to an equidistant sampling rate using cubic spline interpolation. Those equidistant HR
time series were subjected to a fast Fourier transform (FFT) to calculate spectral power in the very low frequency (VLF, 0.02-0.20 Hz), low frequency (0.2-0.6 Hz, reflecting sympathetic and parasympathetic cardiac modulation) and high frequency (HF, 1.0-4.0 Hz, reflecting parasympathetic cardiac modulation) bands. Relative spectral powers were calculated from absolute spectral powers as percentage of total power. Baroreflex sensitivity was determined using the sequence technique. Spectral analysis and baroreceptor-heart rate reflex testing were performed using HemoLab software, freely available from (http://www.haraldstauss.com/HaraldStaussScientific/products/default.html).

Intake Behaviors: Food, water, and 0.15 mol/L NaCl intakes and urine excretion were measured daily using metabolism cages (Nalgene, Rochester, NY) for 4 days during each time point. The first 2 days of measurements were considered acclimatization period and these data were not included. Animals received ad libitum access to standard powdered chow (Harlan Teklad), water and 0.15 mol/L NaCl drink solution (two-bottle choice). Bottle positions were alternated daily to avoid side biases. Urine was collected during day 3 and 4 and used for atomic absorption spectrophotometry (Instrumentation Laboratories), melting-point (Fiske) determinations of electrolyte and osmolyte load and copeptin ELISA assay (USCN Life science Inc.). Protocols were performed according to the manufacturer’s protocol.

Indirect calorimetry: Resting metabolic rate was assessed by indirect calorimetry. Briefly, room air was drawn through a water-jacketed, 2L chamber (Ace Glass, Vineland, NJ) maintained at 30°C (thermoneutrality), and oxygen (S-3A/II, AEI Technologies) and carbon dioxide (CD-31, AEI Technologies) concentrations were determined in effluent air. Data were recorded and analyzed using PowerLab (ADInstruments) and the associated Chart software.

Blood analysis: Blood concentration of sodium, potassium, and glucose were performed using a clinical blood analyzer i-STAT (Abbott). A few drops of blood were collected from each mouse using cheek punch. Whole blood was pipetted in a Chem8+ cartridge and immediately measured using i-STAT analyzer, according to its protocol.

Molecular analyses: Brains were collected from each mouse at the end of the study and were instantly frozen in liquid nitrogen. The entire brain was later frozen in tissue freezing medium (tissue-tek® O.C.T. Compound, Sakura Finetek). In a cryostat, specific brain nuclei (subfornical organ, arcuate nucleus, paraventricular nucleus and cortex) were isolated using a brain punch kit (Stoelting) following the coordinates and landmarks described previous. Isolated nuclei were collected in lysate buffer RTL (All prep RNA/DNA Minikit, Qiagen). Total RNA and genomic DNA (gDNA) were extracted using a RNA/DNA Minikit (Qiagen) following the manufacturer’s protocol with concentrations determined using a NanoDrop ND-1000. cDNA was generated from total RNA by RT-PCR using SuperScript III (Invitrogen) and qRT-PCR was performed using TaqMan gene expression assays (Life Technologies): AT1aR (Mm01166161_m1) and GAPDH (4352932E). The extracted gDNA was used for a genomic DNA PCR. Primers were designed to produce a 2448bp band for the intact fragment of AT1aR gene or a 400bp band for the recombined fragment, where exon-3 of the AT1aR gene was excised (Figure 1B). PCR was performed using Taq Platinum (Invitrogen) following the manufacturer’s protocol.
Images: Photomicrographs of the ROSA\textsuperscript{TdTomato} brains injected with ICV AdCRE or AdGFP were obtained from 40 µm thick sections. Sections were cut from 16 brains (total 4 AdGFP and 12 AdCRE). 1 AdGFP and 3 AdCRE mice were perfusion-fixed with 4% paraformaldehyde every week from 1 to 4 weeks after ICV virus injection. Brains were collected and post-fixed for 24 hours at 4°C in the same fixative and transferred to 30% sucrose at 4°C until sectioning. The mounted sections were covered with VECTASHIELD Mounting Medium for fluorescence with DAPI and refrigerated in the dark. Photos were obtained using a Nikon fluorescence microscope and SFO images were merged using Adobe Photoshop CS (version 8.0).
Supplemental Reference List


Supplemental Figures

Figure S1. Validation of AT_{1a}R Deletion by genomic DNA PCR. Evidence of Cre-mediated recombination in the cortex, paraventricular nucleus (PVN), subfornical organ (SFO) and arcuate nucleus (ARC) measured by PCR of genomic DNA from micropunches. Intact gene = 2448Kb, recombined gene = 400Kb. M=PCR marker (1Kb Plus DNA ladder, Invitrogen). N=negative control.
Figure S2. Validation of SFO-specific targeting: evaluation of other brain regions one week after AdCRE. Additional images from the organum vasculosum of the lamina terminalis (OVLT), medial preoptic nucleus (MnPO), supraoptic nucleus (SON), arcuate nucleus (ARC), area postrema (AP), and the paraventricular nucleus (PVN) of ROSA<sup>TdTomato</sup> mice injected with AdCRE, one week after ICV injection.
Figure S3. Validation of SFO-specific targeting technique: assessment of other brain regions after two, three and four weeks of AdCRE injection. Additional images from the organum vasculosum of the lamina terminalis (OVLT; 2 weeks after AdCRE), the paraventricular nucleus (PVN; 2-4 weeks after AdCRE) and subfornical organ (SFO; 2-4 weeks after AdCRE) of ROSA<sup>tdTomato</sup> mice injected with AdCRE.
Figure S4. Effects of AT_1aR deletion in the SFO on baroreflex sensitivity in DOCA-salt hypertension. Baroreflex gain during DOCA-salt treatment in AdGFP and AdCRE mice compared to their baselines. N=5, each.