Central Mineralocorticoid Receptors and the Role of Ang II and Glutamate in the PVN of Rats with Ang II-Induced Hypertension

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Short title: Ang II and glutamate in PVN of hypertensive rats
Methods

Animals and Diets
Six to 7 weeks old male Wistar rats were obtained from Charles River Breeding Laboratories, Montreal, Quebec, Canada. The rats were housed in a climatized room on a 12-h light/dark cycle at constant room temperature and humidity, and provided with a standard commercial rat chow (120 µmol Na+/ gram) and tap water ad libitum. Animals were acclimatized for 1 week prior to surgeries. All experiments were carried out according to the guidelines of the Canadian Council on Animal Care, which conform to National Institutes of Health guidelines and were approved by the University of Ottawa Animal Care and Use Committee.

Surgical Procedures
For all surgeries, rats were anesthetized with 2% isoflurane in oxygen. For major surgeries (icv and PVN cannulation) 0.1 mg/kg of slow release Buprenorphine was administered sc 40-60 minutes before the start of procedure which is sufficient for 3 days. For minor surgeries (arterial cannulation, sc minipump implantation ) one sc injection (0.04 mg/kg) of regular Buprenorphine was administered pre-surgery.

Osmotic minipumps (Alzet®, model 2002) filled with Ang II at 150 or 500 ng/kg/min were implanted subcutaneously for a 2 week infusion. In sham groups, rats were implanted with pumps filled with saline. One week after sc pump implantation, intra-cerebral cannulations of the lateral ventricle and PVN were performed. A guide cannula (23 gauge) was positioned bilaterally 0.5 mm above the PVN according to the rat atlas of Paxinos and Watson, 1.8 mm posterior to the bregma, 0.4 mm lateral to the sagittal suture, and 7.9 mm ventral from the surface of the skull. For intracerebroventricular infusions, a guide cannula was positioned above the left lateral cerebral ventricle at 0.4 mm posterior to the bregma, 1.4 mm lateral to the sagittal suture, and 3.5 mm ventral to the dura. Guide cannulas were secured to the skull with two jeweler’s screws and acrylic cement and closed with stainless steel obturators. On day 12-13 of sc infusion, the left femoral artery was cannulated with polyethylene-50/10 tubing filled with heparin (1,000 U/ml in 0.9% NaCl). For experiments with intravenous (iv) infusions, the left femoral vein was also cannulated. Arterial and venous catheters were tunneled subcutaneously and externalized through a small puncture in the skin on the back of the neck. The rats were kept in a quiet room over night and the next morning BP was measured without any premedication.

Vehicles and Drugs
Artificial cerebrospinal fluid (121 NaCl, 3.4 KCl, 1.2 MgCl₂, 0.6 NaH₂PO₄, 29 NaHCO₃, and 3.4 glucose mmol/L, pH 7.4, and osmolarity 296 mosmol/kg H₂O) was used as vehicle for eplerenone (acetonitrile and aCSF: 2:98 ratio) and for candesartan and kynurenate (dimethyl sulfoxide and aCSF: 2:98 ratio). Icv infusion of eplerenone was performed at 20 µg/ 3.8 µL for 1 min, followed by 0.83 µg/ 3.8 µL for 60 min. This dose (70 µg total) was based on our preliminary studies assessing doses of eplerenone needed to reverse the hypertension from sc infusion of Ang II at 500 ng/kg/min (data not shown). Infusion of candesartan (0.5 µg/300 nL/min) or kynurenate (0.14 µg/300 nL/min) was performed bilaterally in the PVN (5 or 1.4 µg total on each side) for 10 min. These doses were adapted from our previous study showing that the hypertension from high salt diet in Dahl S rats was reversed by candesartan or kynurenate in...
the PVN\textsuperscript{3}. All compounds were purchased from Sigma or received as gifts (see acknowledgements).

Statistical Analysis

Values are expressed as means ± SE. For comparisons of the area under the curve (AUC) or peak responses among treatments and rat strains, a one or two-way ANOVA was used followed by a Student-Newman-Keuls post hoc multiple comparison. For testing peak changes from baseline, paired \(t\)-tests were used. To test the time-course of changes from baseline, a one-way ANOVA with repeated measures followed by Dunnett’s test was used. The level of significance was set at \(p < 0.05\).

Statistical Analyses for the data in Figures 1-6 in Manuscript

Fig. 1. By one-way ANOVA, responses in MAP and HR were significantly different between treatments in rats with sc infusion of Ang II at 150 ng/kg/min (MAP: \(F=23.8\); HR: \(F=14.0\)); [vehicle vs. kynurenate (MAP and HR: \(P<0.05\)); vehicle vs. candesartan (MAP: \(P<0.05\)); vehicle vs. kynurenate after candesartan (HR: \(P<0.05\)) and Ang II at 500 ng/kg/min (MAP: \(F=36.9\); HR: \(F=25.0\)); [vehicle vs. kynurenate or candesartan (MAP: \(P<0.001\); HR: \(P<0.05\)); vehicle vs. candesartan after kynurenate (HR: \(P<0.05\)); kynurenate or candesartan vs. same treatment without prior infusion (MAP: \(P<0.001\)).

Fig. 2. By one-way ANOVA of the area under the curve, MAP and HR responses were significantly different between treatments (MAP: \(F=56.9\); HR: \(F=45.4\)); [vehicle vs. candesartan or kynurenate (MAP and HR: \(P<0.05\)); candesartan vs. candesartan after kynurenate or kynurenate vs. kynurenate after candesartan (MAP: \(P<0.05\)). By one-way ANOVA with repeated measures, MAP and HR responses to kynurenate were significantly different from vehicle from 2-4 min for MAP and from 8-10 min for HR. Candesartan was significantly different from vehicle from 2-4 min for MAP and from 8-10 min for HR.

Fig. 3. By one-way ANOVA with repeated measures both doses of kynurenate decreased MAP (\(F=22.4\) and 23.1) and HR (\(F=34.9\) and 24.9), all \(p<0.001\). MAP and HR responses to kynurenate were significant different from baseline from 4-6 min for MAP and from 6-8 min for HR.

Fig. 4. By two-way ANOVA, responses in MAP were significantly different between treatments, time points and the interaction between treatments and time points (\(F=41.7, 43.5, 43.9\)); [icv eplerenone with sc Ang II after 1 h vs. 16h (\(P<0.001\)); icv eplerenone with sc Ang II after 16 h vs. all other treatments after 1 or 16 h (\(P<0.001\))]. By one-way ANOVA, responses in MAP from icv infusion of eplerenone with sc infusion of Ang II were significantly different between time points after eplerenone infusion (\(F=48.6\)); [1 vs. 24 h after eplerenone (\(P<0.05\)); 16 vs. 24 h after eplerenone (\(P<0.001\)]).

Fig. 5. By two-way ANOVA, MAP was significantly different between treatments, time points and the interaction between treatments and time points (\(F=17.0, 41.1, 19.2\)); [before eplerenone: icv infusion of eplerenone with sc vehicle vs. others (\(P<0.001\)); 16 h after eplerenone: icv infusion of eplerenone with sc vehicle or Ang II vs. iv infusion of eplerenone with sc Ang II (\(P<0.001\)); before vs. 16 h after icv infusion of eplerenone with sc Ang II (\(P<0.001\))].
Fig. 6. By one-way ANOVA of the area under the curve, MAP and HR responses were significantly different between treatments (MAP: F=28.3; HR: F= 10.5); [candesartan in the PVN after icv vehicle vs. others (MAP: P<0.001); candesartan in the PVN after icv vehicle or eplerenone vs. kynurenate after candesartan in the PVN after icv vehicle or eplerenone (HR: P<0.05). By one-way ANOVA with repeated measures, MAP responses to candesartan in the PVN after icv vehicle were significantly different from others from 4-6 min. For HR, responses to candesartan in the PVN after icv vehicle or eplerenone were significantly different from others from 8-10 min. For peak response data, by one-way ANOVA, MAP and HR responses were significantly different among treatments (MAP: F=31.7; HR: F=17.1); [candesartan in the PVN after icv vehicle vs. others (MAP: P<0.001); candesartan in the PVN after icv vehicle or eplerenone vs. kynurenate after candesartan in the PVN after icv vehicle or eplerenone (HR: P<0.01).
References


Table S1: Baseline MAP and HR levels prior to the first treatment in experimental groups.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>n</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without icv or iv infusion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle, kynurenate in PVN, sc Ang II (150 ng/kg/min)</td>
<td>5</td>
<td>131±2*</td>
<td>430±12</td>
</tr>
<tr>
<td>Vehicle, kynurenate in PVN, sc Ang II (500 ng/kg/min)</td>
<td>4</td>
<td>170±1* a</td>
<td>456±25</td>
</tr>
<tr>
<td>Candesartan, kynurenate in the PVN, sc Ang II (150 ng/kg/min)</td>
<td>4</td>
<td>133±3*</td>
<td>462±18</td>
</tr>
<tr>
<td>Candesartan, kynurenate in the PVN, sc Ang II (500 ng/kg/min)</td>
<td>5</td>
<td>175±11* a</td>
<td>436±6</td>
</tr>
<tr>
<td>Kynurenate, candesartan in the PVN, sc Ang II (500 ng/kg/min)</td>
<td>4</td>
<td>172±4* a</td>
<td>455±10</td>
</tr>
<tr>
<td><strong>With icv or iv infusion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Icv eplerenone, sc vehicle</td>
<td>4</td>
<td>120±6</td>
<td>441±11</td>
</tr>
<tr>
<td>Icv eplerenone, sc Ang II (500 ng/kg/min)</td>
<td>5</td>
<td>180±6* a</td>
<td>425±22</td>
</tr>
<tr>
<td>Iv eplerenone, sc Ang II (500 ng/kg/min)</td>
<td>4</td>
<td>172±3* a</td>
<td>463±23</td>
</tr>
<tr>
<td>Icv veh., cand., kyn. in PVN, sc Ang II (500 ng/kg/min)</td>
<td>4</td>
<td>180±6* a</td>
<td>447±14</td>
</tr>
<tr>
<td>Icv epler., cand., kyn. in PVN, sc Ang II (500 ng/kg/min)</td>
<td>5</td>
<td>177±6* a</td>
<td>439±30</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; * p<0.05 vs. sc vehicle, a p<0.05 vs. groups with sc Ang II (150 ng/kg/min)
Figure S1

Schematic representation of infusion sites inside the PVN, plotted according to the location of dye circles from bilateral injections of 100 nL Evans Blue dye. Infusions into the PVN were performed after sc infusion of Ang II at 500 ng/kg/min for 2 weeks. Symbols connected by broken lines represent bilateral infusion sites from different experimental groups inside the PVN. f indicates fornix; 3V, third ventricle.
Resting MAP and HR after bilateral infusion of candesartan or kynurenate after candesartan in the PVN following icv infusion of vehicle or eplerenone for 1 hour and sc Ang II at 500 ng/kg/min for 2 weeks. Infusion of the first blocker in the PVN was performed 16 h after start of icv infusion of vehicle or eplerenone. The second blocker was infused 20-30 min after start of the first blocker. Values are mean ± SEM.

By two-way ANOVA, MAP was significantly different between treatments, time points and the interaction between treatments and time points (F= 17.1, 13.2, 11.9); [16 h after icv vehicle and before candesartan vs. all others (P<0.001)]. * p<0.001 vs. others.