Hypertensive Dog Plasma Inhibits the Na\textsuperscript{+}-K\textsuperscript{+} Pump of Cultured Vascular Smooth Muscle

HENRY W. OVERBECK, EARL T. WALLICK, RIEKO SHIKUMA, AND WELLS W. MAGARGAL

SUMMARY We investigated the effects of plasma from dogs with perinephritic hypertension on the Na\textsuperscript{+}-K\textsuperscript{+} pump of cultured dog vascular smooth muscle cells. We also measured \textsuperscript{[3H]}ouabain binding by myocardium and vascular tissue. Fresh, unprocessed plasma from healthy dogs during the first 6 weeks of benign one-kidney, one wrapped hypertension and from paired normotensive control dogs was layered over confluent primary cultured puppy aortic smooth muscle cells that had been sodium-loaded with monensin. In 26 paired assays of plasma from four pairs of dogs, cells incubated in the presence of plasma from hypertensive dogs had significantly reduced total (\textit{p} < 0.01) and ouabain-sensitive (\textit{p} < 0.001) \textsuperscript{86}Rb\textsuperscript{+} uptakes, but their intracellular sodium content did not differ from cells incubated in paired normotensive plasma. We no longer detected these uptake differences when passaged cells or cells cocultured with bovine endothelial cells were used for assay or when plasma was treated with protease inhibitors or boiled. However, boiled plasma increased the sodium content of the assay cells, suggesting an ionophore-like effect. Levels of pump inhibitory activity in plasma appeared to remain constant during Weeks 1 to 6 of hypertension. We found no evidence for altered numbers of pump sites in cardiovascular tissues from these hypertensive dogs. These findings support the hypothesis that plasma factors inhibit the membrane Na\textsuperscript{+}-K\textsuperscript{+} pump in vascular smooth muscle cells in this form of hypertension. These plasma inhibitory factors apparently do not induce pump molecules. (Hypertension 12: 32-38, 1988)

KEY WORDS • ouabain-sensitive rubidium-86 uptake • cell sodium content • cell ion transport • cultured vascular smooth muscle • Na\textsuperscript{+},K\textsuperscript{+}-adenosine triphosphatase • \textsuperscript{[3H]}ouabain binding

THERE is increasing evidence that plasma factors inhibit Na\textsuperscript{+}-K\textsuperscript{+}-adenosine triphosphatase (ATPase) in hypertension. We recently reported that fresh, unprocessed plasma from rats with benign one-kidney, one clip hypertension reduces ouabain-sensitive \textsuperscript{86}Rb\textsuperscript{+} uptake of primary cultured rat aortic smooth muscle cells when compared with cells incubated in normotensive plasma. This abnormality occurs in the very early (<1 week) and also chronic (>3 weeks) stages of the hypertension. The assay system we used has many advantages over previous methods: The assay involves vascular muscle cells, a postulated effector cell for the putative humoral inhibitor in hypertension; intracellular ion concentrations are easily measured, facilitating data interpretation; and use of unprocessed plasma avoids creation, modification, release, or destruction of inhibitory or stimulatory factors by acidification, boiling, or freezing. There is evidence for humoral pump inhibitors in dogs with chronic one-kidney, one wrapped (1K1W) perinephritic hypertension. However, the best evidence to date involves boiled plasma supernates assayed in rat tail artery strips. In the present study, we used our homologous cultured cell assay to reexamine this form of hypertension. Fresh, unprocessed plasma obtained within 60 minutes of bleeding was layered over cultured dog aortic smooth muscle cells, and effects on \textsuperscript{86}Rb\textsuperscript{+} uptake were measured. To detect temporal changes in levels of humoral inhibitor, blood was assayed repeatedly over the first 6 weeks of hypertension. To detect endothelial cell modulation of the inhibition, we also assayed with cocultures. Finally, because there is evidence that humoral inhibitors in hypertensive rats may induce Na\textsuperscript{+}-K\textsuperscript{+} pump molecules in vascu-
For Readers in the United States

☑ Yes, I want to receive my personal copy of

☐ Circulation
☐ Circulation Research
☐ Stroke
☐ Hypertension
☐ Arteriosclerosis
☐ Recurring Bibliography of Hypertension
☐ Recurring Bibliography of Hypertension

Institutional rates available upon request.

PLEASE PRINT

Name ____________________________
Address ____________________________
City ____________________________ State ______ Zip ______
My specialty ____________________________

Please send my subscription beginning with the ________ issue.

Advance payment required before copies are sent.

☐ Send bill
☐ Check enclosed, payable to the American Heart Association.
☐ This is a renewal. My account number is ________________

OFFER ENDS NOVEMBER 1, 1988

For Readers Outside the United States, Japan, and Europe

☑ Yes, I want to receive my personal copy of

☐ Circulation
☐ Circulation Research
☐ Stroke
☐ Hypertension
☐ Arteriosclerosis
☐ Recurring Bibliography of Hypertension
☐ Modern Concepts

Institutional rates available upon request.

PLEASE PRINT

Name ____________________________
Address ____________________________
City ____________________________ State ______ Zip ______ Country ______
My specialty ____________________________

Please send my subscription beginning with the ________ issue.

Advance payment required before copies are sent.

☐ Send bill
☐ Check enclosed, payable to the American Heart Association.
☐ This is a renewal. My account number is ________________

OFFER ENDS NOVEMBER 1, 1988

American Heart Association

Please send a sample copy of:

☐ Arteriosclerosis
☐ Circulation
☐ Circulation Research
☐ Hypertension
☐ Stroke
☐ Recurring Bibliography of Hypertension

to the librarian at my institution

with my recommendation to subscribe.
lar muscle, we measured binding of [H]ouabain to cardiovascular tissues of these dogs.

Materials and Methods

[^8]RbCl and [H]ouabain were obtained from New England Nuclear (Boston, MA, USA). Ouabain, Type 1 elastase, and monensin were from Sigma Chemical (St. Louis, MO, USA). Collagenase (CLS II) was from Cooper Biomedical (Parsippany, NJ, USA). Fetal calf serum was from Hyclone Laboratories (Logan, UT, USA) or Flow Laboratories (McLean, VA, USA). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Grand Island, NY, USA). Endothelial cell growth factor was from Sigma Chemical or Meloy Laboratories (Springfield, VA, USA). All other chemicals were the best grade commercially available.

Vascular Smooth Muscle Cultures

Dog aortic smooth muscle cells were isolated by a modification of our published procedures for rat aorta. Briefly, aortas were excised aseptically from pentobarbital-anesthetized mongrel puppies obtained from the pound. The excised aortas were placed in HEPES-buffered Hanks' balanced salt solution, cleansed of fat, connective tissue, and blood, and opened longitudinally. The intima was scraped with a spatula to remove endothelium, and the adventitia was removed. Isolated medial tissue was minced with scissors into 2-mm² pieces and digested with collagenase/elastase as described for rat aorta.

Isolated cells were plated at 1.5 x 10^5/cm² in DMEM and 5% fetal calf serum. Plating efficiency was 50 to 70%. Cells were maintained in 95% air, 5% CO₂ and fed twice per week. Primary cells were used at confluence between 7 and 14 days after isolation. Cells were subcultured at 1:3 or 1:5 split ratio and used at confluence up to the sixth passage.

Bovine Endothelial Cells and Cocultures

Cells were isolated as previously described for porcine endothelial cells. Bovine aortas were obtained from a local slaughterhouse. Endothelial cells were removed from the lumen of cleansed aortas by digestion with collagenase, 0.33 mg/ml HEPES-buffered Hanks' balanced salt solution, at 37 °C for 20 minutes. Isolated cells were plated on T25 flasks and grown in DMEM plus 10% fetal calf serum, with endothelial cell growth factor, 20 μg/ml, and heparin, 90 μg/ml. Cells at confluence were passaged at a 1:2 ratio. Cocultures of dog aortic smooth muscle cells and bovine endothelial cells were prepared as follows: Primary dog smooth muscle cells at 50 to 60% confluence on 35-mm dishes were seeded with 1 x 10^5 subcultured (Passage 2-5) endothelial cells. Subcultured dog smooth muscle cells were plated at 2 x 10^5 cells/35-mm dish; after 24 hours 1 x 10^5 endothelial cells were added to the dish. All cocultures were used 2 to 3 days after adding the endothelial cells.

Production of Hypertension

Using methods we have previously described, we surgically prepared dogs in pairs; one had perinephritic hypertension and the other was an appropriate normotensive control. Briefly, we trained healthy male mongrel pound dogs, weighing 18 to 27 kg and maintained on standard dog chow and water ad libitum, to lie quietly for measurement of baseline conscious arterial pressure by femoral artery puncture. Dogs with baseline mean arterial pressures in excess of 130 mm Hg were rejected.

In 14 randomly selected dogs, which composed the 1K1W hypertensive group, we made a left flank incision with the dogs under pentobarbital anesthesia (Nembutal, 25 mg/kg i.v.) and sterile conditions. We dissected the left kidney free of its fat pad and wrapped it in silk with an outer layer of silicone sheeting (0.005 in. thick, Dow Corning, Midland, MI, USA) to minimize adhesions. Through the same incision, we removed the right kidney. In 14 randomly selected, paired, normotensive control dogs, we simply dissected the left kidney free of its fat pad and either removed the right kidney (n = 7) or left it intact (n = 7). Postoperatively, all dogs received 400,000 units of penicillin and 0.5 mg of streptomycin i.m. daily (Combiotic, Pfizer) for 5 successive days. We measured femoral arterial pressure, body weight, hematocrit, plasma sodium and potassium (flame emission; Model IL 683, Instrumentation Laboratories, Lexington, MA, USA), and plasma creatinine (creatinine determination kit, Sigma) one to three times weekly in all dogs, and we carefully monitored general health postoperatively. At necropsy we weighed the heart and examined other organs, including kidneys, for gross abnormalities. Procedures followed throughout the study were in accordance with institutional guidelines.

Plasma

We obtained arterial blood from conscious dogs one to three times per week by femoral arterial puncture. Paired hypertensive and control normotensive dogs were bled in random order, and after an aliquot was taken for determination of hematocrit, citrate (50 μl 0.2 M Tris; citrate/ml blood) was added to the blood. The concentration of citrate was carefully controlled because of known effects on cell Rb⁺ uptake. In some cases protease inhibitors (aprotinin, 1 U/ml blood; bacitracin, 0.4 mM final concentration; and diisopropyl fluorophosphate, 0.1 mM final concentration) were also added to the paired blood samples. These levels of protease inhibitors in plasma had no effect on Rb⁺ uptake by cultured dog or rat vascular smooth muscle cells. The blood was iced immediately, and all subsequent manipulations were done at 4 °C. The blood was centrifuged for 10 minutes at 3000 rpm, and the supernatant was recentrifuged at 20,000 rpm for 15 minutes. The resulting platelet-poor plasma was assayed within 60 minutes of bleeding.
Boiled plasma supernates were prepared as follows: Citrated blood was centrifuged at 3000 rpm for 10 minutes at 4 °C. Plasma was then incubated at room temperature for 20 minutes and centrifuged at 20,000 rpm for 20 minutes at 25 °C. The supernate was placed in boiling water for 5 minutes and centrifuged at 10,000 rpm for 90 minutes. The resulting supernate was assayed immediately. This procedure is a modification of that developed by Gruber and Buckalew and subsequently used by Pampanini et al.

**Rb** Uptake Assay

Plasmas from the paired normotensive control and hypertensive dogs were assayed by an investigator who did not know the plasma source. As in our previous study, we used a modification of the procedure of Brock et al. except that the preincubation time in plasma before adding **RbCl** was increased to 15 minutes and ouabain-insensitive uptake was measured in the presence of 0.5 mM ouabain. Plasma was added at a concentration of 80% to media bathing the cells, as previously, and incubation with **RbCl** was 15 minutes at 37 °C (uptake linear for at least 30 minutes). Intracellular Na+ and **Rb** were measured as previously described. **Rb** uptake was expressed as nanomoles per minute per milligram of protein.

In most assays monensin, from a stock solution of 40 mg/ml 100% ethanol, was added to plasma or boiled plasma supernates to achieve a final plasma concentration of 40 μg/ml. Pilot experiments (n = 3) indicated that ouabain-sensitive **Rb** uptake by dog smooth muscle was maximal at monensin concentrations exceeding 30 μg/ml plasma. At a monensin level of 40 μg/ml, the increment in uptake over baseline levels in the absence of monensin was 90%; accompanying increases in cell sodium averaged 43%. Increases of plasma monensin concentrations above 40 μg/ml resulted in further increases in cell sodium content but no increase in ouabain-sensitive **Rb** uptake by the cells. Thus, in these assays involving monensin, cell sodium concentrations were not rate-limiting for the Na+-K+ pump.

Additionally, the high baseline pump activity increased chances for detection of pump inhibition.

**Preparation of Membranes and Measurement of [3H]Ouabain Binding**

Six paired hypertensive (4–6 weeks of hypertension) and control dogs were shipped to the University of Cincinnati. After the dogs were killed (within 1 week after arrival in Cincinnati), hearts were removed, trimmed free of atria and connective tissue, and weighed. The tissue was then cut into small pieces, minced with scissors, and homogenized in an Omnimixer (DuPont, Wilmington, DE, USA) with two volumes (2 ml/g tissue) of a solution containing 30 mM Tris maleate (pH 7) and 0.3 M sucrose. Samples of the homogenate were used for measurement of [3H]ouabain binding. The remainder of the homogenate was used to prepare microsomes essentially as described by Van Alstyne et al.

Homogenates and microsomes were similarly prepared from abdominal aorta, femoral and mesenteric arteries and from femoral and mesenteric veins. Protein was measured by the method of Lowry et al. and [3H]ouabain binding was performed for 30 minutes at 37 °C in the presence of 5 mM MgCl₂, 5 mM Tris phosphate, 50 mM Tris hydrochloride (pH 7.4), 10⁻⁷ M [3H]ouabain (approximately 10,000 Ci/mol) as previously described. Nonspecific binding was estimated as just described, but in the additional presence of 10⁻⁴ unlabeled ouabain. ATPase activities were determined as previously described.

**Statistics**

We analyzed results by the paired or unpaired two-tailed Student's t test and by linear regression, rejecting the null hypothesis at a p level below 0.05. Data for each experiment were analyzed both on the basis of assays irrespective of dogs (n value = number of paired assays) and on the basis of averaged values of assay differences within each pair of dogs (n value = number of pairs of dogs).

**Results**

**General Outcome**

All dogs remained in good health throughout the study, and necropsy revealed no gross abnormalities other than perinephritis. Hypertension was defined as a sustained rise in blood pressure over preoperative values. Table 1 lists body weights, femoral mean arterial blood pressures, and plasma sodium, potassium, and creatinine concentrations averaged over the duration of the hypertension for the 14 pairs of dogs studied. Final heart weights were available in only six normotensive dogs, the remaining dogs having undergone procedures to create cardiac hypertrophy in an unrelated terminal study. Paired Student's t test revealed no significant differences in body weight and plasma sodium concentrations between the paired hypertensive and normotensive dogs. Trends for increases in heart weight reached statistical significance when heart weight was normalized for body weight, as in our previous studies. Hypertensive dogs had rises averaging 35% (p < 0.001) in mean arterial pressure, decreases averaging 8% (p < 0.02) in hematocrit, increases averaging 8% (p < 0.01) in plasma potassium concentration, and rises averaging 33% (p < 0.001) in plasma creatinine concentration. In no hypertensive dog did average postoperative creatinine concentration exceed 1.65 mg/dl, or roughly double normal levels. The 7 two-kidney, and 7 one-kidney control dogs were also compared; no significant differences were observed in any of these variables or in the effect of plasma on cell **Rb** uptake or cell sodium, as will be described.
### Table 1. Characteristics in 14 Pairs of Hypertensive and Normotensive Dogs

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Hypertensive</th>
<th>$D \pm SE$</th>
<th>$p$</th>
<th>Normotensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt (kg)</td>
<td>22.95</td>
<td>0.50 ± 1.05</td>
<td>&gt;0.5</td>
<td>23.45</td>
</tr>
<tr>
<td>Heart wt (g)</td>
<td>202.9</td>
<td>—</td>
<td>&lt;0.1*</td>
<td>164.2</td>
</tr>
<tr>
<td>Heart wt (g)/body wt (kg)</td>
<td>8.84</td>
<td>—</td>
<td>&lt;0.05*</td>
<td>7.00</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>39.1</td>
<td>3.5 ± 1.3</td>
<td>&lt;0.02</td>
<td>42.6</td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>142.1</td>
<td>36.5 ± 4.0</td>
<td>&lt;0.001</td>
<td>105.6</td>
</tr>
<tr>
<td>Plasma Na$^+$ (mEq/L)</td>
<td>139.4</td>
<td>0.4 ± 0.3</td>
<td>&gt;0.2</td>
<td>139.0</td>
</tr>
<tr>
<td>Plasma K$^+$ (mEq/L)</td>
<td>4.03</td>
<td>0.30 ± 0.04</td>
<td>&lt;0.001</td>
<td>3.73</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dl)</td>
<td>1.20</td>
<td>0.30 ± 0.06</td>
<td>&lt;0.001</td>
<td>0.90</td>
</tr>
</tbody>
</table>

*Unpaired t test; 14 hypertensive and six normotensive dogs. $D = mean$ difference.

### $^{86}$Rb$^+$ Uptake Assays

Sixty-two paired assays in 10 pairs of postoperative dogs involved confluent primary dog aortic smooth muscle cells. The results of these assays are presented in Table 2. In 26 assays in four pairs of dogs, incubation of monensin-treated cells with unprocessed plasma from hypertensive dogs resulted in highly significant ($p < 0.001$) decreases in cell ouabain-sensitive $^{86}$Rb$^+$ uptake, as compared with cells incubated in the paired normotensive plasma. In cells incubated in hypertensive plasma, there were also significant reductions in total uptake and, additionally, trends of borderline statistical significance toward increases in ouabain-insensitive uptakes. In contrast, there were no significant differences in sodium content of cells incubated in hypertensive or normotensive plasma. For this experiment, data presented in Table 2 were calculated on the basis of averaged differences observed in individual pairs of dogs, but calculations on the basis of differences in individual paired assays led to similar conclusions. Additionally, analysis of data separately for one pair of dogs in which 11 assays were done also indicated significant ($p < 0.01$) reductions in ouabain-sensitive uptakes in cells incubated in hypertensive plasma.

To detect endothelial-cell modulation of this inhibitory effect, 15 assays involved dog primary aortic smooth muscle cells cocultured with passage bovine endothelial cells seeded at ratios of 1:1 ($n = 13$), 1:5 ($n = 1$), and 5:1 ($n = 1$) and treated with monensin. Assays of unprocessed plasma samples from one pair of dogs over the 6 weeks of hypertension revealed increases in variability as compared with experiments with smooth muscle alone, but no evidence for significant differences in any variable studied or in results using the various coculture ratios (see Table 2).

In an attempt to enhance the significant differences we had observed in the initial experiment, we performed a third experiment involving primary cells and boiled plasma supernates. Table 2 presents the results of 21 paired assays in five pairs of dogs involving boiled plasma supernates and monensin-treated cells. The results indicate that

### Table 2. Rubidium Uptake and Intracellular Sodium Concentration

<table>
<thead>
<tr>
<th>Group</th>
<th>Total $^{86}$Rb$^+$ uptake (nmol/min/mg)</th>
<th>Ouabain-insensitive</th>
<th>Ouabain-sensitive</th>
<th>[Na$^+$] (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary cells, monensin, unprocessed plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 4 pairs; 26 assays)*</td>
<td>48.25</td>
<td>4.07</td>
<td>44.18</td>
<td>9.16</td>
</tr>
<tr>
<td>Normotensive</td>
<td>46.39</td>
<td>4.18</td>
<td>42.20</td>
<td>8.93</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>1.87 ± 0.18</td>
<td>0.11 ± 0.04</td>
<td>1.98 ± 0.14</td>
<td>0.23 ± 0.33</td>
</tr>
<tr>
<td>Mean difference ± SE</td>
<td>&lt;0.01</td>
<td>≥0.05</td>
<td>&lt;0.001</td>
<td>≥0.5</td>
</tr>
<tr>
<td>Significance ($p$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary cocultured cells, monensin, unprocessed plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 15 assays; 1 pair)†</td>
<td>42.98</td>
<td>3.45</td>
<td>39.53</td>
<td>17.00</td>
</tr>
<tr>
<td>Normotensive</td>
<td>43.90</td>
<td>3.47</td>
<td>40.49</td>
<td>15.60</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>0.92 ± 1.84</td>
<td>0.04 ± 0.27</td>
<td>0.96 ± 1.80</td>
<td>1.39 ± 0.87</td>
</tr>
<tr>
<td>Mean difference ± SE</td>
<td>&gt;0.5</td>
<td>&gt;0.5</td>
<td>&gt;0.5</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Significance ($p$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary cells, monensin, boiled plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 5 pairs; 21 assays)*</td>
<td>50.71</td>
<td>3.06</td>
<td>47.66</td>
<td>21.47</td>
</tr>
<tr>
<td>Normotensive</td>
<td>51.12</td>
<td>3.24</td>
<td>47.88</td>
<td>22.47</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>0.41 ± 0.59</td>
<td>0.19 ± 0.11</td>
<td>0.22 ± 0.63</td>
<td>1.29 ± 1.57</td>
</tr>
<tr>
<td>Mean difference ± SE</td>
<td>&gt;0.5</td>
<td>&gt;0.1</td>
<td>&gt;0.5</td>
<td>&gt;0.4</td>
</tr>
<tr>
<td>Significance ($p$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data analyzed on basis of average differences in each pair of dogs.
†Data analyzed on basis of assays.
boiling obliterated, rather than enhanced, differences between hypertensive and normotensive plasma. Boiled plasma supernates also increased the sodium content of the monensin-treated cells by an additional 145% (p < 0.001) without significantly elevating their $^{86}$Rb$^+$ uptakes (p > 0.5); this is further evidence that cell sodium was not pump rate-limiting in our monensin-treated assay cells.

Sixty-three paired assays of plasma from eight pairs of dogs involved passaged dog aortic smooth muscle cells alone or cocultured with passaged bovine endothelial cells. These experiments represented an attempt to duplicate the positive results just described by using passaged cells that are more easily prepared. However, results were uniformly negative and will only be briefly reported. Nineteen assays of plasma from three pairs of dogs involved unprocessed plasma, no protease inhibitors, no monensin (ouabain-sensitive uptakes: normotensive = 19.9; 1K1W = 18.6; difference $\bar{D} \pm SE = 1.3 \pm 0.7$ nmol/min/mg; p > 0.10). Nine assays of plasma from three pairs involved monensin-treated muscle cells and unprocessed plasma. There was also no significant difference (p > 0.2) in the sodium content of the monensin-treated cells by 138%, compared with incubation in unprocessed plasma (see Table 2) revealed no evidence for changes in the level of humoral inhibitor during the 6-week duration of hypertension. There was also no significant correlation between magnitude of hypertension and level of inhibition ($r = 0.065$, p > 0.05).

\[ ^{3}H \text{Ouabain Binding to Membrane Fractions} \]

Yields of protein per gram of wet weight in homogenate and microsomal fractions from cardiac and vascular tissues were similar in normotensive and hypertensive dogs. In mesenteric artery, for example, yield of homogenate fraction averaged $15.2 \text{ mg protein/g wet weight}$ in normotensive dogs compared with $16.6 \text{ mg protein/g wet weight}$ in hypertensive dogs ($n = 6$ pairs). The average yield of microsomal protein from this homogenate in normotensive dogs was 9.4% and in hypertensive dogs, 8.2%.

Binding of $^{3}H$Ouabain to both the homogenate and the microsomal fractions was linear with protein (data not shown). Even in the homogenate, specific binding was easily detected. There was no significant difference (p > 0.2) in $^{3}H$Ouabain binding to membranes from normotensive and hypertensive dogs in any of the six tissues studied (Table 3). There was also no significant difference (p > 0.2) between hypertensive (8.8 $\pm$ 2.4 $\mu$mol/mg/hr) and normotensive (11.3 $\pm$ 2.8 $\mu$mol/mg/hr) dogs (5 pairs) in ouabain-sensitive Na$^+$-K$^+$-activated ATPase activity in cardiac ventricular microsomes.

**Discussion**

Increasing evidence suggests the presence of humoral inhibitors of the Na$^+$-K$^+$ pump in several forms of hypertension. In 1K1W hypertension in dogs, we reported that in vitro ouabain-sensitive

---

**Table 3.** $^{3}H$Ouabain Binding to Homogenate and Microsomes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Binding (pmol/mg protein)</th>
<th>Microsomes</th>
<th>Homogenate</th>
<th>Hypertensive</th>
<th>Normotensive</th>
<th>Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal aorta</td>
<td>0.43 $\pm$ 0.08</td>
<td>0.41 $\pm$ 0.05</td>
<td>0.06 $\pm$ 0.15</td>
<td>1.47 $\pm$ 0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral artery</td>
<td>0.30 $\pm$ 0.08</td>
<td>0.48 $\pm$ 0.07</td>
<td>0.65 $\pm$ 0.20</td>
<td>0.99 $\pm$ 0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>0.45 $\pm$ 0.11</td>
<td>0.49 $\pm$ 0.16</td>
<td>2.05 $\pm$ 0.42</td>
<td>2.63 $\pm$ 0.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral vein</td>
<td>0.18 $\pm$ 0.06</td>
<td>0.39 $\pm$ 0.21</td>
<td>0.64 $\pm$ 0.23</td>
<td>0.89 $\pm$ 0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric vein</td>
<td>0.29 $\pm$ 0.04</td>
<td>0.48 $\pm$ 0.13</td>
<td>1.06 $\pm$ 0.20</td>
<td>1.17 $\pm$ 0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac muscle</td>
<td>3.72 $\pm$ 1.29</td>
<td>3.56 $\pm$ 0.71</td>
<td>29.3 $\pm$ 5.4</td>
<td>29.1 $\pm$ 7.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE from four to six pairs of dogs (p > 0.2).
$^{86}\text{Rb}^+$ uptake by mesenteric arteries and veins is decreased. There was some evidence that depression of uptake may have been greater when the arterial strips from hypertensive dogs were incubated in the dog's own unprocessed plasma, in contrast to incubation in crossover normotensive plasma. However, decreased uptake was still present in vessels incubated in Krebs-Henseleit buffer. It was later reported by another laboratory that supernates of boiled plasma from such dogs suppress, by 40%, ouabain-sensitive $^{86}\text{Rb}^+$ uptake of tail arteries from normal rats used for assay. According to the investigators, the boiling had presumably prevented breakdown of the inhibitor substances. In neither reported study was cell sodium measured or the role of endothelium assessed. In both studies dogs were in the chronic (>4 weeks) stage of hypertension.

The purposes of the present experiments were several: 1) to demonstrate the presence of inhibitor in unprocessed plasma not subjected to boiling or freezing procedures; 2) to assay dog plasma in an appropriate homologous system, cultured dog vascular smooth muscle cells; 3) to clarify the role of cell sodium in the pump activity observed; 4) to explore the relationship of duration of hypertension and levels of inhibitor; 5) to determine if endothelial cells modify the pump inhibition; and 6) to investigate whether circulating inhibitors induce Na⁺-K⁺ pump molecules in cardiovascular tissue.

The results of the present experiments achieved these purposes. Unprocessed plasma from hypertensive dogs has small, but highly significant, inhibitory effects on the activity of the Na⁺-K⁺ pump of dog vascular smooth muscle cells. These pump inhibitory effects are not associated with changes in the sodium content of the cells used for assay, so they cannot be related to induced decreases in cell sodium concentration. Plasma levels of the inhibitory factor apparently remain fairly constant during the first 6 weeks of the hypertension. In contrast to assays involving pure smooth muscle cells, assay of plasma with cocultured endothelial and muscle cells appears to eliminate the inhibitory effect of hypertensive plasma, although these experiments involved only one pair of dogs (15 assays), and the results require further verification. Additionally, treatment of plasma by boiling or by addition of protease inhibitors also apparently eliminates the inhibitory effect. Finally, [3H]ouabain binding provided no evidence that the circulating inhibitor induces pump molecules in cardiovascular tissues of the hypertensive dogs.

Total (p < 0.01) and ouabain-sensitive (p < 0.001) uptakes by the cells used for assay were significantly reduced in the presence of hypertensive plasma. The reductions in total and ouabain-sensitive uptakes were detected despite plasma potassium concentrations in hypertensive dogs averaging 8% higher than those in normotensive dogs. Increases in plasma creatinine in hypertensive dogs were too small, and the dogs too healthy, to consider a role for uremia in this pump inhibition. The lower postoperative hematocrit in hypertensive dogs probably reflects volume expansion accompanying this form of hypertension.

Results of this assay were similar to those we have reported for assay of plasma from one-kidney, one clip hypertensive rats using a similar homologous cultured cell system (but in the absence of monensin). Both hypertensive and normotensive rat plasmas increased Na⁺-K⁺ pump activity in the cultured rat aortic muscle cells (the hypertensive plasma induced less of an increase, however), compared with the effects of balanced salt solution alone. The plasma effect was associated with significant elevations in cell sodium content, probably reflecting mitogen-induced increases in cell sodium-proton exchange.

Thus, as in our previous experiment, the lesser increase in pump activity of the assay cells in the presence of hypertensive dog plasma could indicate a lower concentration of plasma stimulator(s) of the pump. However, we believe this explanation is unlikely, because assay cell sodium was not higher in the presence of normotensive plasma, as would be expected if higher concentrations of, for example, mitogens or angiotensin, were present. Furthermore, cell sodium was not rate-limiting for the pump in our assay system.

Boiling of plasma or treatment with protease inhibitors clearly did not enhance, and appeared to eliminate, the inhibitory effect of hypertensive plasma. This result conflicts with that of Pamnani et al., who found that boiled plasma supernates reduced ouabain-sensitive $^{86}\text{Rb}^+$ uptake of rat tail artery strips by about 40%. Although boiling procedures were similar, the two studies differ in that Pamnani et al. froze the supernates for future assay, whereas we assayed immediately. Freezing, in addition to boiling, may create or release pump-inhibitory substances. The two studies also differ in that Pamnani et al. used a nonhomologous assay system, did not verify that cell sodium was not rate-limiting in their assay cells, and did not measure the effect of cell sodium levels on their results. It is noteworthy that our experiments suggest that boiling plasma may create or release ionophores or stimulators of transport systems that increase assay cell sodium content.

Assay with passaged cells appeared to be less sensitive than assay with primary cells. It seems possible that the change in cell phenotype with passage is accompanied by deletion of critical membrane receptor or regulating sites.

We detected fairly constant levels of inhibitor throughout the first 6 weeks of canine perinephritic hypertension. Similarly, in one-kidney, one clip hypertension in rats, the levels of inhibitor appear during the first week and persist unchanged into the established phases (3–4 weeks) of hypertension. The time course of plasma-induced inhibition may
differ in other forms of experimental hypertension. For example, in rats with deoxycorticosterone acetate-salt hypertension, Buckalew (personal communication, 1987) found evidence for higher levels of humoral digitalislike factor in early hypertension, which diminished as the hypertension became chronic. Similarly, Songu-Mize et al.\(^1\) reported evidence for humoral pump inhibitors in prehypertensive deoxycorticosterone acetate-salt rats, which disappeared as benign hypertension became established.

In hypertension in rats, the humoral inhibitor may induce Na\(^+\)-K\(^+\) pump molecules in arterial smooth muscle cells,\(^2-6\) accounting for the elevated levels of activity of the Na\(^+\)-K\(^+\) pump in vascular tissue in vitro observed by most investigators.\(^5\) In contrast, as noted, our previous in vitro studies of vascular tissue from dogs with chronic perinephritic hypertension indicated decreased Na\(^+\)-K\(^+\) pump activity.\(^4\) This finding suggests that the humoral inhibitor in this form of hypertension or species (or both) may not induce Na\(^+\)-K\(^+\) pump molecules. In this regard, measurement of \([\text{H}]\text{ouabain binding to homogenates and microsomal membranes from cardiac ventricles and several conduit arteries and veins in the present investigation provided no statistical evidence for altered numbers of pump sites in tissues from hypertensive dogs (although trends were present suggesting the possibility of slight increases). Similarly, myocardial Na\(^+\),K\(^+\)-ATPase activity was unchanged. Although more than 6 weeks’ exposure to the humoral inhibitor may be necessary for induction of pump molecules, or study of more than four to six pairs of dogs may be necessary to reveal small but significant increases in numbers of pump sites, it is more likely that the low level of inhibition (averaging about 4.5%) was insufficient for induction, given the high reserve capacity of the Na\(^+\)-K\(^+\) pump.

Acknowledgments

We are grateful to Bessie A. Ingram for her carefully conducted assays. We appreciate the surgical assistance of Robert E. Blackmon, the help of Jeanne D. Bonner with tissue cultures, and the secretarial help of April D. Sandlin.

References


14. Smith JB, Rozengurt E. Serum stimulates the Na\(^+\),K\(^+\) pump in quiescent fibroblasts by increasing Na\(^+\) entry. Proc Natl Acad Sci USA 1978;75:5560-5564


Hypertensive dog plasma inhibits the Na+-K+ pump of cultured vascular smooth muscle.
H W Overbeck, E T Wallick, R Shikuma and W W Magargal

doi: 10.1161/01.HYP.12.1.32

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 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/12/1/32