Ouabain Binding and Na\textsuperscript{+} Content in Resistance Vessels and Skeletal Muscles of Spontaneously Hypertensive Rats and K\textsuperscript{+}-Depleted Rats

CHRISTIAN AALKJÆR, KELD KJELDSEN, AAGE NØRGAARD, TORBEN CLAUSEN, AND MICHAEL J. MULVANY

SUMMARY The possible role of Na\textsuperscript{+} in the development of hypertension in rats was explored in measurements of intracellular Na\textsuperscript{+}, \textsuperscript{22}Na efflux, and \textsuperscript{3}H-ouabain binding sites in resistance vessels and skeletal muscles. In resistance vessels obtained from 13-week-old spontaneously hypertensive rats (SHR) or age-matched Wistar-Kyoto rats (WKY), (Na), total or ouabain-resistant \textsuperscript{22}Na efflux, and the concentration of \textsuperscript{3}H-ouabain binding sites showed no significant differences. Soleus muscles obtained from 6-week-old and 13-week-old SHR contained 5 to 11\% more \textsuperscript{3}H-ouabain binding sites than those of WKY. The small difference in ouabain binding probably was related more to variations in growth rate and strain than to the hypertension. In SHR and WKY the Na\textsuperscript{+} and K\textsuperscript{+} contents of gastrocnemius muscles were almost identical at 6 and 13 weeks of age. By contrast, in Wistar rats in which the (Na), of skeletal muscle was increased sixfold by K\textsuperscript{+} depletion, the systolic blood pressure was decreased by 10\%. The K\textsuperscript{+} depletion was associated with a 35 to 55\% decrease in the concentration of \textsuperscript{3}H-ouabain binding sites in both resistance vessels and skeletal muscles. The results provide no support for any simple cause-effect relationships between either elevated (Na), or altered concentration of \textsuperscript{3}H-ouabain binding sites and hypertension in SHR. (Hypertension 7: 277–286, 1985)

KEY WORDS • ouabain binding • Na\textsuperscript{+}-K\textsuperscript{+} pump • sodium content • resistance vessels • muscle • potassium deficiency • SHR

It has been repeatedly proposed that hypertension is associated with and possibly caused by increased Na\textsuperscript{+} content, Na\textsuperscript{+} permeability, and altered Na\textsuperscript{+} transport in vascular smooth muscle cells.\textsuperscript{1–5} The present study was initiated to test this possibility by investigating the Na\textsuperscript{+} metabolism of small arteries from spontaneously hypertensive rats (SHR) and from their genetically related normotensive controls, Wistar-Kyoto rats (WKY). The arteries had internal diameters around 200 \(\mu\)m, small enough to be considered as resistance vessels.\textsuperscript{6} In these experiments we measured the intracellular Na\textsuperscript{+} concentration — (Na), — and \textsuperscript{22}Na efflux. Also, as increased Na\textsuperscript{+} turnover may lead to adaptation of the concentration of Na\textsuperscript{+}-K\textsuperscript{+} pumps, we measured the concentration of \textsuperscript{3}H-ouabain binding sites in these vessels. Because the small size of these vessels prevented precise measurements, as a control of the results on the small vessels, we investigated the Na\textsuperscript{+} and K\textsuperscript{+} contents and made a detailed analysis of the binding of \textsuperscript{3}H-ouabain in skeletal muscles using established methods.\textsuperscript{7,8} The use of skeletal muscles for this purpose has an additional advantage in that as skeletal muscles contain the largest depot of K\textsuperscript{+} in the body, an altered K\textsuperscript{+} homeostasis in this tissue might influence Na\textsuperscript{+}-K\textsuperscript{+} homeostasis in other tissues. Finally, as another check on the possible relation of increased (Na), to hypertension, we investigated Na\textsuperscript{+} metabolism, \textsuperscript{3}H-ouabain binding, and blood pressure in rats in which the (Na), had been elevated by the administration of a K\textsuperscript{+}-deficient diet. As we have previously reported that this diet causes a decrease in the number of \textsuperscript{3}H-ouabain binding sites in skeletal muscle,\textsuperscript{9,10} we investigated whether a similar change occurred in smooth muscle cells.
Materials and Methods

Animals

All experiments were performed with male and female, 6- to 7-week-old or 12- to 14-week-old SHR and age- and sex-matched WKY supplied by Møllégård breeding center (L. Skensved, Denmark) and male and female 12-week-old Wistar rats from our own colony. The K⁺ depletion was induced by maintaining the animals on K⁺-deficient fodder containing 0.75 mmol K and 152 mmol Na/kg dry wt. All other animals were kept on standard fodder containing 262 mmol K and 87 mmol Na/kg dry wt. The systolic blood pressure was measured with a tail cuff in caged anesthetized rats. The characteristics of the rats used are shown in Table 1. Compared with the WKY, all SHR had significantly increased blood pressure (22% at age 6 weeks and 45% at age 13 weeks), increased heart/body weight ratios, but lower body weight. In contrast, the K⁺-depleted Wistar rats had blood pressures that were 10% less than the control Wistar rats despite an increased heart/body weight ratio.

Solutions, Chemicals, and Isotopes

The incubation medium (standard buffer) for the measurement of (Na) and 22Na efflux in small arteries was, as in earlier studies, a modified Krebs-Ringer bicarbonate buffer containing (mM): 119 NaCl, 25 NaHCO₃, 4.7 KCl, 1.18 KH₂PO₄, 1.17 MgSO₄, 1.6 CaCl₂, 0.026 ethylenediaminetetraacetic acid (EDTA), and 5.5 D-glucose, pH 7.4. For measurement of (Na) in small arteries from K⁺-depleted rats, a low K⁺ buffer was used, which was similar to standard buffer except that K⁺ was replaced by Na⁺ to give 1.4 mM K⁺; this level corresponded to the K⁺ concentration in the plasma of such animals. A lithium buffer was used for the washout of 22Na (standard buffer in which NaCl and NaHCO₃ had been replaced by 107 mM LiCl and 25 mM Li₂CO₃, respectively). The ³H-ouabain binding to skeletal muscle biopsy was measured with a vanadate tris buffer containing 5.5 mM D-glucose and 1.3 mM CaCl₂, as in earlier studies. The ³H-ouabain binding to skeletal muscle biopsies was measured with a vanadate tris buffer containing 10 mM tris chloride, 3 mM MgSO₄, 1 mM tris vanadate, and 250 mM sucrose, pH 7.2. All bicarbonate buffers were gassed continuously with a mixture of 5% CO₂ and 95% O₂; the vanadate tris buffer was agitated with air throughout the experiment.

The standard rat fodder was from Korn og Foderstof Kompagniet, Aarhus, Denmark, and the K⁺-deficient fodder was from the Altromin Co., Lage, West Germany. All chemicals were of analytical grade. Ouabain was obtained from the Merck Co., Darmstadt, West Germany, Na from Amersham International Ltd., England, and ³H-ouabain from New England Nuclear Corporation, Boston, Massachusetts. The purity of ³H-ouabain was checked by the Na⁺-K⁺-ATPase extraction method. The radiopurity was found to be 93%, and this was corrected for in the calculations of the specific ³H-ouabain binding.

Table 1. Characteristics of the Rats Used

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of animals</th>
<th>Sex</th>
<th>Age (wk)</th>
<th>Body wt (g)</th>
<th>Heart/body wt (mg/g)</th>
<th>SBP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>23</td>
<td>Male</td>
<td>13.6 ± 0.1</td>
<td>301 ± 6†</td>
<td>3.41 ± 0.04§</td>
<td>170 ± 3§</td>
</tr>
<tr>
<td>WKY</td>
<td>23</td>
<td>Male</td>
<td>13.5 ± 0.1</td>
<td>317 ± 5</td>
<td>2.90 ± 0.04</td>
<td>117 ± 2</td>
</tr>
<tr>
<td>SHR</td>
<td>10</td>
<td>Male</td>
<td>6.2 ± 0.1</td>
<td>116 ± 3‡</td>
<td>—</td>
<td>126 ± 5‡</td>
</tr>
<tr>
<td>WKY</td>
<td>10</td>
<td>Male</td>
<td>6.2 ± 0.1</td>
<td>134 ± 5</td>
<td>—</td>
<td>103 ± 6</td>
</tr>
<tr>
<td>SHR</td>
<td>4</td>
<td>Female</td>
<td>6.3 ± 0.0</td>
<td>111 ± 4</td>
<td>—</td>
<td>123 ± 4‡</td>
</tr>
<tr>
<td>WKY</td>
<td>4</td>
<td>Female</td>
<td>6.3 ± 0.0</td>
<td>129 ± 6</td>
<td>—</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>K⁺-depleted Wistar*</td>
<td>10</td>
<td>Female</td>
<td>12.3 ± 0.2</td>
<td>204 ± 5</td>
<td>3.02 ± 0.05§</td>
<td>108 ± 2‡</td>
</tr>
<tr>
<td>Control Wistar</td>
<td>10</td>
<td>Female</td>
<td>12.3 ± 0.2</td>
<td>218 ± 6</td>
<td>2.77 ± 0.05</td>
<td>120 ± 3</td>
</tr>
<tr>
<td>Control Wistar</td>
<td>4</td>
<td>Male</td>
<td>12.3 ± 0.2</td>
<td>302 ± 6</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* K⁺ depletion was induced by maintaining the rats on K⁺-deficient fodder for 4 weeks.

SBP = systolic blood pressure; SHR = spontaneously hypertensive rats; WKY = Wistar-Kyoto rats.

† p < 0.05.

‡ p < 0.01.

§ p < 0.005.

Values are means ± SEM.
media of vessels from Wistar rats, 12 vessels — 6 from K⁺-depleted and 6 from control rats — were fixed in glutaraldehyde and embedded in Epon, and longitudinal sections were made with the use of standard histological methods. In these sections the smooth muscle cells were seen in cross section, and the content of smooth muscle cells in the media was determined to be 74 ± 1% and 71 ± 2% (p > 0.10) in K⁺-depleted and control Wistar rats respectively.

After determination of cell volume the vessels were prewashed for 60 minutes in K⁺-free buffer at 37°C, incubated for 30 minutes at 37°C in K⁺-free buffer containing 1 × 10⁻⁶ M ³H-ouabain (18 Ci/mmol), and finally washed for 120 minutes in a series of tubes containing K⁺-free buffer at 0°C. The ³H-ouabain retained in the vessels and in the washout medium was counted by liquid scintillation spectrometry (Mark III, model 6880, Searle Analytic Inc.). Washout curves were constructed by sequentially adding the counts in the washout media in reverse order to the counts in the vessels. The activity of the total uptake of ³H-ouabain was estimated as the intercept with the ordinate of the back-extrapolated linear portion of the efflux curve. Parallel experiments were carried out to determine the nonspecific uptake of ³H-ouabain by adding 1 × 10⁻¹ M unlabeled ouabain to the incubation medium. The specific binding was determined as the difference between the activity measured in the absence and in the presence of 1 × 10⁻³ M ouabain and expressed as picomoles per milliliter of smooth muscle cell.

Measurements of (Na), and (22)Na efflux were made as described previously. In brief, for the determination of (Na), the vessel segments were loaded for 30 minutes at 37°C in standard buffer with ³Na (0.63 Ci/mol) after determination of cell volume. The vessels were then washed through a series of tubes for 45 minutes in lithium buffer at 0°C. The amount of ³Na in the cells at time zero was determined by constructing washout curves and extrapolating the late straight part of the efflux curves back to time zero and are expressed as millimoles per liter of smooth muscle cell. For the determination of ³Na efflux the vessels were loaded in ³Na as just described and then washed for 15 minutes through a series of tubes containing standard buffer at 37°C. From the washout curves the mean rate constant was determined between 9 and 15 minutes after the onset of washout in the absence of ouabain (total rate constant) and with 1 × 10⁻³ M ouabain added after 9 minutes (ouabain-resistant rate constant).

³H-ouabain Binding to Soleus Muscle and (Na), and (K), of Gastrocnemius Muscle

Standard in vitro measurements of the ³H-ouabain binding site concentration were carried out in biopsies or both lateral strips of the soleus muscles. Biopsies weighing 2 to 8 mg were prepared and equilibrated with ³H-ouabain (1 × 10⁻⁶ M, 1.8 Ci/mmole) in vanadate trifluor es buffer at 37°C. In other samples the lateral muscle strips were prepared and equilibrated with ³H-ouabain (2 × 10⁻⁶ M, 0.3 Ci/mmole) in K⁺-free Krebs-Ringer bicarbonate buffer at 30°C. For both types of preparations the equilibration with ³H-ouabain (2 × 60 minutes) was followed by a washout at 0°C (4 × 30 minutes) to remove ³H-ouabain from the extracellular space. In each experiment a set of samples was incubated with the addition of 1 × 10⁻⁴ M ouabain to determine nonspecific ³H-ouabain uptake. In other experiments the accuracy of the standard procedure was investigated with the variations indicated.

In vivo experiments were performed by injecting rats intraperitoneally with 1 ml/100 g body weight of a 154 mM NaCl solution containing either 15 µCi/ml ³H-ouabain and 1.25 mM unlabeled ouabain (standard procedure) or 5 µCi/ml ³H-ouabain and 0.075 mM unlabeled ouabain. After 60 minutes the animals were killed by decapitation. Blood samples were collected from the neck vessels into glass tubes. Plasma was obtained by centrifugation (1500 g for 10 min) and taken for ³H-activity determination to ensure that adequate absorption of the injected ouabain had taken place. Soleus muscles were immediately dissected out and washed and washed for 4 × 30 minutes at 0°C in K⁺-free buffer.

Following the 4-× 30-minute cold wash, muscles or muscle biopsies from in vitro or in vivo experiments were blotted, weighed, and homogenized in 2 ml of 5% trichloroacetic acid (TCA). After centrifugation (1500 g for 10 min) 1 ml of the clear supernatant was taken for liquid scintillation counting (Mark II, model 6848, Searle Analytic Inc.) of the ³H-activity. On the basis of the specific activity of the incubation medium (in vitro) or the medium injected (in vivo), muscle wet weight and the ³H-activity of the samples, the amount of ³H-ouabain retained after the cold wash was calculated. For the in vitro experiments the ³H-ouabain binding site concentration (picomoles per gram of wet weight) was then calculated by first subtracting the unspecific ³H-ouabain uptake (1–5%) and then multiplying with correction factors to allow for (1) incomplete saturation and (2) for loss of specifically bound ouabain during washout. For the biopsy preparations these factors were determined to be (1) 1.14 and 1.13 for SHR and WKY respectively, on the basis of Scatchard plot analysis (see Figure 5B). For SHR and WKY respectively, from washout curves (see Figure 4). For all muscle strip preparations the correction factors previously determined for Wistar muscle strip were used: (1) 1.12, (2) 1.17. For biopsies from Wistar rats and for the in vivo experiments corrections were done as described elsewhere. All ³H-ouabain binding site concentrations reported are corrected values.

The Na⁺ and K⁺ contents of gastrocnemius muscles were determined by flame photometry (FLM3, Radiometer, Copenhagen, Denmark) on muscle samples taken immediately after killing the animals. On the basis of the observation that K⁺ depletion was not associated with any change in water content or ¹⁴C-sucrose space in skeletal muscle, a water content of 74% and an extracellular space of 12.7% were used for the calculation of (Na), and (K).
Statistics

Values given in the text, tables, and figures generally are presented as arithmetic means ± SEM (n = number of observations). As (Na), and the rate constants for 22Na efflux showed logarithmic distribution, however, these values are given as geometric means with 95% confidence intervals in brackets. Differences between observed means generally were tested for significance with an unpaired two-tailed t test. In Table 4 differences in 3H-ouabain binding site concentration in skeletal muscle were tested with two-way analysis of variance. Because the 3H-ouabain binding site concentration was calculated as the difference between total and nonspecific uptake (see the previous section), the variance of the 3H-ouabain binding site concentration was calculated taking into account the variances of both the measured total uptake and the measured nonspecific uptake.

Results

Effects of Hypertension

Small Arteries

Figure 1 shows the time course of 22Na washout into ice-cold lithium buffer of small arteries of SHR and WKY in the experiments in which (Na), was determined. The rate constants for the slow phase of this washout (between 9 and 45 minutes) were 0.0059 ± 0.0006 min⁻¹ (n = 23) and 0.0056 ± 0.0006 min⁻¹ (n = 24) for vessels obtained from WKY and SHR respectively and were not significantly different. Values for (Na), in these vessels are shown in Figure 2. The distribution was similar in the two groups, and there was no significant difference in (Na), (Table 2).

In another series of experiments, 22Na efflux was determined at 37°C. After an initial rapid efflux presumably caused by washout of extracellular and extravascular 22Na, a slow and almost constant efflux rate was obtained in vessels from both SHR and WKY. This efflux was considered a transmembrane efflux of intracellular 22Na, and the total rate constant was determined (Table 2). In parallel experiments 1 × 10⁻³ M ouabain was added to the washout medium after 9 minutes and an immediate reduction in the 22Na efflux was seen (Figure 3). The ouabain-resistant rate constant for the 22Na efflux was then determined (Table 2), and this too was similar in the vessels of SHR and WKY. In further experiments with four vessels from SHR and four vessels from WKY, the efflux medium was changed to K⁻⁻⁻-free buffer after 9 minutes instead of adding ouabain. This change induced the same decrease in the efflux rate constant in both groups as did the addition of 1 × 10⁻³ M ouabain, which indicates that 1 × 10⁻³ M ouabain was sufficient to inhibit the ouabain-sensitive efflux.

Determination of 3H-ouabain binding to small arteries from SHR and WKY showed that the concentration of 3H-ouabain binding sites was not significantly different (Table 2). In a separate experiment, with small arteries from four SHR and four WKY, the incubations were done with 2 × 10⁻⁶ M 3H-ouabain (i.e., twice the standard concentration). This change did not increase the amount of bound 3H-ouabain, which suggests that in both groups of rats 1 × 10⁻⁶ M 3H-ouabain may be sufficient to achieve saturation.
Table 2. Na⁺ Metabolism of 200 μm Mesenteric Resistance Vessels from 13-week-old Spontaneously Hypertensive Rats, Wistar-Kyoto Rats, K⁺-Depleted Wistar Rats, and Control Wistar Rats

<table>
<thead>
<tr>
<th></th>
<th>SHR</th>
<th>WKY</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Na), (mmol/L cell)</td>
<td>14.8 [13.9–15.7] (24/4)</td>
<td>14.1 [13.1–15.2] (23/4)</td>
<td>&gt;0.60</td>
</tr>
<tr>
<td>Total 22Na efflux rate constant (min⁻¹)</td>
<td>0.130 [0.121–0.140] (10/10)</td>
<td>0.128 [0.119–0.138] (8/8)</td>
<td>&gt;0.80</td>
</tr>
<tr>
<td>Ouabain resistant 22Na efflux rate constant (min⁻¹)</td>
<td>0.061 [0.054–0.066] (28/10)</td>
<td>0.060 [0.055–0.065] (26/10)</td>
<td>&gt;0.80</td>
</tr>
<tr>
<td>Concentration of ³H-ouabain binding sites (pmol/ml cell)</td>
<td>250 ± 28 (13/5)</td>
<td>259 ± 39 (13/5)</td>
<td>&gt;0.80</td>
</tr>
</tbody>
</table>

Table 3 shows the tissue content of Na⁺ and K⁺ in gastrocnemius muscle from 6-week-old and 12-week-old SHR and WKY. There was no difference between SHR and age-matched WKY, nor did the contents change appreciably with age.

The concentration of ³H-ouabain binding sites in soleus muscles from SHR and WKY was measured with the procedures indicated (Table 4). The mean value measured in the SHR preparations was between 5 and 11% larger than that of the WKY preparations, and analysis of variance showed that for all groups taken together, this difference was significant (p < 0.001). As we have previously observed that the concentration of ³H-ouabain binding sites in normal Wistar rats decreases markedly from the onset of maturity, it was of particular interest to examine wheth-

**Figure 3.** Washout of ²²Na from small arteries from 13-week-old SHR and normotensive WKY. After loading in standard buffer containing ²²Na for 30 minutes at 37°C, washout was performed through a series of vials each containing 2 ml of buffer for 15 minutes at 37°C. After a 9-minute washout, 1 × 10⁻³ M ouabain was added to the washout medium. The efflux is expressed as the instantaneous rate constant, which is the rate constant calculated for each 2-minute washout period. Each closed circle represents the mean of observations on 28 vessels obtained from 10 SHR, and each open circle is the mean of observations on 26 vessels obtained from 10 WKY. In a parallel series of experiments with 10 vessels from 10 SHR and 9 vessels from 9 WKY, the washout was continued without the addition of ouabain. The rate constants between 9 and 15 minutes of these experiments are shown as closed and open triangles, SHR and WKY respectively. The bars denote SEM.

**Skeletal Muscle**

Table 3 shows the tissue content of Na⁺ and K⁺ in gastrocnemius muscle from 6-week-old and 12-week-old SHR and WKY. There was no difference between SHR and age-matched WKY, nor did the contents change appreciably with age.

The concentration of ³H-ouabain binding sites in soleus muscles from SHR and WKY was measured with the procedures indicated (Table 4). The mean value measured in the SHR preparations was between 5 and 11% larger than that of the WKY preparations, and analysis of variance showed that for all groups taken together, this difference was significant (p < 0.001). As we have previously observed that the concentration of ³H-ouabain binding sites in normal Wistar rats decreases markedly from the onset of maturity, it was of particular interest to examine wheth-

**Table 3. Tissue Contents of Na⁺ and K⁺ in Gastrocnemius Muscle from Spontaneously Hypertensive Rats and Normotensive Wistar-Kyoto Rats**

<table>
<thead>
<tr>
<th>Strain (age)</th>
<th>Na⁺ content (μmol/g wet wt)</th>
<th>p</th>
<th>K⁺ content (μmol/g wet wt)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR (6 wk)</td>
<td>25 ± 1 (6)</td>
<td>&gt;0.10</td>
<td>113 ± 3 (6)</td>
<td>&gt;0.40</td>
</tr>
<tr>
<td>WKY (6 wk)</td>
<td>21 ± 2 (6)</td>
<td></td>
<td>116 ± 2 (6)</td>
<td></td>
</tr>
<tr>
<td>SHR (13 wk)</td>
<td>22 ± 1 (8)</td>
<td>&gt;0.40</td>
<td>107 ± 1 (8)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>WKY (13 wk)</td>
<td>21 ± 1 (8)</td>
<td></td>
<td>107 ± 3 (8)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM with the number of animals in parentheses.

SHR = spontaneously hypertensive rats, WKY = Wistar-Kyoto rats.
er the development of hypertension was associated with anomalies in the population of $^3$H-ouabain binding sites. The small difference between SHR and WKY seemed unaffected by age, however, and between 6 and 13 weeks of age the concentration of $^3$H-ouabain binding sites decreased by 38%, which is not different from the 37% decrease observed in normal Wistar rats.

As it has been suggested that hypertension is associated with inhibition of the $\text{Na}^+\text{-K}^+$ pump by a circulating inhibitor,\textsuperscript{21,22} our analysis included experiments designed to reveal the influence of such a factor. First, the preparations were washed for $2 \times 60$ minutes before the incubation with $^3$H-ouabain to allow the release of ligands occupying the ouabain receptor. This procedure produced no significant increase in the concentration of $^3$H-ouabain binding sites (Table 4), which is in keeping with earlier observations on Wistar preparations.\textsuperscript{10} Second, when $^3$H-ouabain binding was allowed to take place in vivo with the use of an established procedure for normal Wistar rats,\textsuperscript{17} the soleus muscles of SHR and WKY were found to contain the same concentration of $^3$H-ouabain binding sites as measured in vitro, which indicates that the binding of ouabain is not interfered with by a circulating ouabain-like factor (Table 4). Third, as ouabain at the saturating concentrations used might have displaced an inhibitor, the in vivo experiments were repeated with $^3$H-ouabain that was 17 times less concentrated (0.075 mM). At this subsaturating concentration the muscles in the SHR still bound more $^3$H-ouabain than those of the WKY, which gives no indication of a higher concentration of a ouabain-like factor in the plasma of the SHR than in the WKY.

To identify possible systematic sources of error and at the same time determine the kinetic characteristics of the $^3$H-ouabain binding, a further series of experiments was performed that compared biopsies of soleus muscles from SHR and WKY aged 13 weeks. To test whether the binding of $^3$H-ouabain took place under conditions where the distribution of ouabain in muscle biopsies had reached a steady state, the $^3$H-activity accumulated after 60 or $2 \times 60$ minutes of incubation in vanadate tris buffer containing $1 \times 10^{-5}$ M $^3$H-ouabain was measured. In biopsies obtained from SHR the uptake corresponded to $516 \pm 23$ ($n = 8$) and $512$.

### Table 4. $^3$H-Ouabain Binding in Skeletal Muscles from Spontaneously Hypertensive and Normotensive Wistar-Kyoto Rats

<table>
<thead>
<tr>
<th>Age</th>
<th>$^3$H-Ouabain incubation procedure</th>
<th>SHR</th>
<th>WKY</th>
<th>$P_{\text{experiment}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 Weeks</td>
<td>In vitro, standard procedure</td>
<td>522 ± 23 (10/4)</td>
<td>464 ± 31 (10/4)</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td></td>
<td>preceded by $2 \times 60$ min wash</td>
<td>479 ± 17 (15/8)</td>
<td>444 ± 19 (15/8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6 Weeks</td>
<td>In vitro, standard procedure</td>
<td>769 ± 25 (12/6)</td>
<td>698 ± 19 (10/6)</td>
<td>&gt;0.98</td>
</tr>
<tr>
<td></td>
<td>In vivo, standard procedure</td>
<td>756 ± 13 (8/4)</td>
<td>708 ± 15 (8/4)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SEM, with the number of preparations/number of animals in parentheses, and the results are analyzed by two-way analysis of variance ($P_{\text{interaction}} = \text{not significant}$).

SHR = spontaneously hypertensive rats; WKY = Wistar-Kyoto rats.

![Figure 4. Washout of $^3$H-activity from soleus muscle biopsies from 13-week-old SHR and normotensive WKY](http://hyper.ahajournals.org/)

After standard incubation washout was performed for $12 \times 30$ minutes in $2 \text{ml}$ of buffer at $0^\circ$C. At the end of the washout, the $^3$H-activity remaining in the muscle biopsies was determined and, by adding successively the amount of $^3$H-activity released into the washout media, the $^3$H-activity remaining in the muscles at each transfer between the tubes was calculated and expressed as a percentage of the initial level. Each curve represents the mean of six soleus muscle biopsies obtained from each hindlimb of four SHR and four WKY. The bars denote SEM.
± 23 pmol/g wet wt (n = 8, p > 0.80). In WKY the corresponding values were 415 ± 10 (n = 8) and 403 ± 12 pmol/g wet wt (n = 8, p > 0.40). These results indicate that the standard incubation period of 120 minutes was adequate to ensure equilibrium, and that the small difference between the SHR and WKY was not due to differences in the rate of ³H-ouabain binding.

As differences in the rate of ³H-ouabain release during the cold wash following incubations might constitute another source of error, washout experiments as well as experiments without washout were performed. From Figure 4 it can be seen that the late and slow component of the washout of ³H-activity from biopsies of SHR and WKY took place with half-lives of 9.0 and 10.6 hours respectively. These values are in good agreement with those determined from muscle biopsies of Wistar rats. Extrapolation of the late straight portion of the washout curves back to the onset gave values corresponding to 32% of the initial content for both groups of biopsies. When converted to concentration of ³H-ouabain binding sites this intercept level corresponded to 474 ± 13 (n = 8) and 397 ± 11 pmol/g wet wt (n = 8, p < 0.001) for SHR and WKY respectively. In parallel experiments, where washout was omitted, the amount of ³H-ouabain specifically bound corresponded to 533 ± 39 (n = 8) in preparations from SHR and 438 ± 50 pmol/g wet wt (n = 8) in preparations from WKY. These control experiments support the conclusion that the slight increase in the concentration of ³H-ouabain binding sites in the biopsy preparations of the SHR represents a true difference and is not an artifact of the washing procedure.

To test the importance of possible differences in affinity of ouabain for the binding sites, ³H-ouabain binding was measured with ³H-ouabain concentrations in the range of 2.5 × 10⁻⁷ to 5 × 10⁻⁶ M. For technical reasons it was impossible to test a wider range of concentrations: Measurements with lower concentration would require incubation periods exceeding 6 hours with ensuing deterioration of tissue integrity, and at higher concentrations the precision of the measurements becomes increasingly unsatisfactory owing to relatively high nonspecific uptake. As shown in Figure 5A, the specific binding of ³H-ouabain is saturable. The apparent dissociation constants (Kₐ) are

**FIGURE 5.** Effect of the concentration of ouabain on the binding of ³H-ouabain in soleus muscles obtained from 13-week-old SHR and normotensive WKY. The ³H-ouabain binding site concentration was measured in muscle biopsies with the standard incubation conditions except that the incubations took place at 2.5 × 10⁻⁷ to 5 × 10⁻⁶ M ouabain. A. Bound ³H-ouabain (EO) as a function of the concentration of ouabain in the incubation medium (Of). B. Bound (EO) versus bound/free ³H-ouabain (EO/Of). The lines of this Scatchard-type plot have been constructed with the use of regression analysis. The calculated values for the intercepts with the ordinate (EO_max) are given. Each point represents the mean of five observations on average. Soleus muscle biopsies were taken from the hindlimbs of five SHR and five WKY and randomized into groups of four to six samples. The bars denote SEM.
in the range of $10^{-7}$ M, as earlier determined in muscle biopsies from normal Wistar rats. The maximal uptake of $^3$H-ouabain at an infinitely high concentration of ouabain in the incubation medium (EO$_{0}$), as determined from the intercepts of the linear regression lines, was $506 \pm 22$ (n = 11) and $484 \pm 17$ pmol/g wet wt (n = 11) for SHR and WKY respectively. Statistical evaluation showed no difference between the two regression lines of the Scatchard-type plot (r = 0.79 and 0.92 in SHR and WKY respectively; Figure 5B).

Effects of K$^+$ Depletion

The K$^+$ depletion of Wistar rats reduced the number of $^3$H-ouabain binding sites both in the small arteries (by 37%; Table 2) and in soleus muscle (by 55%; Table 5). The K$^+$ depletion caused a 37% increase in the (Na) of the small arteries (measured after equilibration in standard buffer but with the same K$^+$ concentration as the plasma of such animals; Table 2). The K$^+$ depletion also caused a 96% increase in Na$^+$ content and a 33% decrease of K$^+$ content of gastrocnemius muscle (Table 5). From these measurements of Na$^+$ and K$^+$ contents it can be estimated (see Methods) that the (Na), increased 511% while the (K), decreased 35% in gastrocnemius muscle.

Table 5. Effect of K$^+$ Depletion and Sex on the $^3$H-Ouabain Binding Site Concentration in Soleus Muscle Biopsies and the Total Na$^+$ and K$^+$ Contents of Gastrocnemius Muscles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sex</th>
<th>$^3$H-Ouabain binding sites (pmol/g wet wt)</th>
<th>Na$^+$ content ($\mu$mol/g wet wt)</th>
<th>K$^+$ content ($\mu$mol/g wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$^+$ depleted*</td>
<td>Female</td>
<td>$154 \pm 7$ (50/10)</td>
<td>$47 \pm 2$ (10/10)</td>
<td>$68 \pm 2$ (10/10)</td>
</tr>
<tr>
<td>Normal</td>
<td>Female</td>
<td>$344 \pm 9$ (69/10)</td>
<td>$24 \pm 1$ (10/10)</td>
<td>$103 \pm 2$ (10/10)</td>
</tr>
<tr>
<td>Normal</td>
<td>Male</td>
<td>$372 \pm 12$ (15/4)</td>
<td>$23 \pm 1$ (4/4)</td>
<td>$109 \pm 2$ (4/4)</td>
</tr>
</tbody>
</table>

*K$^+$ depletion was induced by maintaining the rats on K$^+$-deficient fodder for 4 weeks

Values are means ± SEM, with the number of observations/number of animals in parentheses.

Discussion

Small Arteries

In this study we were not able to detect any significant changes in the Na$^+$ metabolism of resistance vessels of SHR and WKY as regards their (Na), or their total ouabain-resistant $^{22}$Na efflux. From the efflux measurements it seems likely that there was no difference in Na$^+$ transport (active or carrier mediated or both). Because of the low (Na), and the negative membrane potential the major part of the measured $^{22}$Na efflux was active or carrier mediated or both. This finding suggests that resistance vessels of SHR and WKY have similar Na$^+$ metabolism, but it should be stressed that the (Na), does not necessarily reflect the Na$^+$ activity, which probably is a more important parameter. Such measurements must, however, await the refinement of the intracellular Na$^+$ electrodes now used for measuring Na$^+$ activity in teniae coli (C. Aickin and A. Bradin, personal communication, 1983). The effective Na$^+$ permeability of the plasma membranes of the vessels of SHR and WKY also may be compared. Effective permeability is a function of the influx, the transplasmalemmal electrochemical Na$^+$ gradient, and the cell volume/cell surface area ratio. For the following reasons all three of these parameters appear to be equal in these vessels of SHR and WKY. First, because Na$^+$ efflux is equal, Na$^+$ influx also must be equal under steady state conditions. Second, results from this study indicate that (Na), is equal, and results from previous studies suggest that the membrane potential of mesenteric resistance vessels of SHR and WKY is also equal (H. Nilsson and M.J. Mulvany, 1981, personal communication). Third, we have previously reported that the cell radius in vessels of SHR and WKY is the same, which indicates a similar cell volume/surface ratio. Therefore, the effective permeability of the plasma membranes in the SHR and WKY vessels should be similar. It should be emphasized, however, that because Na$^+$ influx is divided between passive diffusion and exchange fluxes, it is not presently clear whether this conclusion also applies to true Na$^+$ permeability. Although these findings all refer to values measured under in vitro conditions, our further finding of an unaltered $^3$H-oouabain binding site concentration in the vessels of SHR indicates that their capacity for performing active Na$^+$-K$^+$ transport had not changed. This finding suggests to us that the Na$^+$ metabolism of the SHR resistance vessels also was probably close to normal under the conditions prevailing in the intact animal.

Previous investigations of vascular sodium metabolism in SHR have been carried out with aortic and tail artery preparations. Comparison of those studies with our findings is complicated because of the variety of protocols and the different control animals that have been used. The age of the rats investigated also covered a wide range. Nevertheless, as regards the (Na), there does seem to be general agreement. After equilibration in physiological saline at physiological temperature, the (Na), in the aorta and tail artery of SHR and of age-matched normotensive controls is similar, which is in agreement with our findings in resistance vessels. As regards sodium fluxes, though, the situation is less clear. In aorta the sodium efflux is reported to be increased. Furthermore, under conditions in which the (Na), has been greatly increased by incubating preparations in K$^+$-free solutions at low temperature for many hours, the rate of Na$^+$ efflux from the tail artery is also increased in SHR. By contrast, under conditions in which (Na), is closer to phys-
io logical values, the efflux of $^{22}\text{Na}$ from the tail artery of adult SHR is normal, as it was in our experiments with resistance vessels from such animals. Taken together it seems that if the Na$^+$ metabolism of the vasculature of the SHR is altered, it is not found in all vessels and that the divergence from normality must be relatively small. As far as our own experiments are concerned, from the size of the variance in our measurements of total and ouabain-dependent $^{22}\text{Na}$ effluxes it can be shown that there is only a 10% probability of the difference in the $^{22}\text{Na}$ effluxes from resistance vessels of SHR and WKY exceeding about 20%. Similarly, for the measurements of $^3\text{H}$-ouabain binding it can be shown that there is only a 10% probability that the difference exceeds approximately 35%.

**Skeletal Muscle**

Although the Na$^+$ and K$^+$ contents of the gastrocnemius muscles were the same in SHR and WKY, we were able to detect a small (5–11%) increase in the $^3\text{H}$-ouabain binding site concentration. This finding therefore supports the finding in the small vessels that there are no or only small differences in the concentration of ouabain-binding sites. Our finding that the $K_D$ of the $^3\text{H}$-ouabain binding sites was in the range of $10^{-7}$ M is in agreement with other studies of ouabain binding in skeletal muscle and in cardiac muscle. In view of the fact that in rat skeletal muscle the normal age-dependent variation in the $^3\text{H}$-ouabain binding site concentration may amount to several hundred percent, the observed difference in $^3\text{H}$-ouabain binding site concentration in the present study seems negligible. Furthermore, in soleus muscles obtained from age-matched normotensive male Wistar rats and WKY, the $^3\text{H}$-ouabain binding site concentration was found to differ by 19% (see Tables 4 and 5). This finding, together with the observation that the difference in $^3\text{H}$-ouabain binding site concentration between soleus muscle of SHR and WKY could already be detected in the young animals when the increase in blood pressure was small, indicates that the difference is more likely to be due to developmental or strain differences rather than to hypertension. This moderate increase in $^3\text{H}$-ouabain binding site concentration is, however, in contrast to some studies on cardiac muscle sarcometa, in which preparations from SHR were found to contain 40 to 50% fewer $^3\text{H}$-ouabain binding sites than those obtained from WKY. This discrepancy could be related to differences in recovery and preparations. A recent study, however, reported that myocardial preparations from SHR contained 10% more Na$^+$-K$^+$-ATPase activity than those obtained from WKY.

As already discussed, the small difference in $^3\text{H}$-ouabain binding concentration in skeletal muscle between SHR and WKY was less than the precision of the method used for determination of $^3\text{H}$-ouabain binding sites in resistance vessels; therefore, we were not able to determine whether or not the discrepancy represented a difference between skeletal and vascular muscle.

**Ouabainlike Factor**

An endogenous digitalsislike factor has been proposed as a possible pathogenic factor in the development of hypertension. The good agreement between our measurements of $^3\text{H}$-ouabain binding site concentration in skeletal muscle under in vitro conditions with our measurements of $^3\text{H}$-ouabain binding in vivo, as well as with the in vitro measurements after prolonged prewash, provides no support for the existence of such a factor in either SHR or WKY. Such conclusions also were drawn from previous, more extensive comparisons of $^3\text{H}$-ouabain binding in vivo and in vitro in Wistar soleus muscle. This finding therefore indicates that the small difference in $^3\text{H}$-ouabain binding site concentration in soleus muscle of SHR and WKY is not due to different levels of a circulating endogenous ligand showing reversible binding to the $^3\text{H}$-ouabain receptors.

**K$^+$-Depleted Rats**

The finding of a lack of difference between the Na$^+$ metabolism of smooth and striated muscle cells of SHR and WKY is in contrast to the results of our studies of corresponding preparations from the K$^+$-depleted rats. Here, K$^+$ depletion was accompanied by a decrease in the $^3\text{H}$-ouabain binding site concentration both in the resistance vessels and in soleus muscle and by an increase of about 500% in the (Na), of gastrocnemius muscle. It is also probable that the in vivo (Na), of the small arteries was slightly increased, in view of our finding that in vessels from K$^+$-depleted rats that were equilibrated in solutions containing in vivo plasma K$^+$ concentrations (1.4 mM), the (Na), was increased by about 40% (Table 2). Despite these indications of increased vascular (Na), however, the blood pressure of the K$^+$-depleted rats was not increased, indeed it was reduced by 10%. Thus, these experiments with K$^+$-depleted rats also provide no support for any simple cause-effect relationship between altered vascular Na$^+$ metabolism and hypertension.

**Conclusion**

In resistance vessels from 13-week-old SHR no evidence was found for an altered Na$^+$ metabolism compared to vessels from age- and sex-matched WKY. These observations were supported by findings in skeletal muscles. In contrast, in K$^+$-depleted rats, in which there was a decrease in blood pressure, a decrease in $^3\text{H}$-ouabain binding site concentration and an increase in (Na), were found in resistance vessels as well as in skeletal muscles. The results provide no evidence for a simple relationship between vascular Na$^+$ metabolism and hypertension.

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