Cytochemically Assayable Na\textsuperscript{+},K\textsuperscript{+}-ATPase Inhibition by Milan Hypertensive Rat Plasma

SHARON HOLLAND, JACQUELINE MILLETT, JAMSHID ALAGHBAND-ZADEH, HUGH DE WARDENER, PATRIZIA FERRARI, AND GIUSEPPE BIANCHI

SUMMARY The ability of plasma from 3- and 9-week-old Milan hypertensive rats and their normotensive controls to inhibit Na\textsuperscript{+},K\textsuperscript{+}-adenosine triphosphatase (ATPase) was studied using cytochemical bioassay techniques in fresh tissue. With a validated cytochemical bioassay that measures the capacity of biological samples to stimulate glucose-6-phosphate dehydrogenase activity in guinea pig proximal tubules as an indication of their capacity to inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase, the mean glucose-6-phosphate dehydrogenase-stimulating ability of the plasma of the 9-week-old Milan hypertensive rats and their normotensive controls was 586.0 ± 88 and 23.4 ± 8.3 U/ml (n = 7; p<0.001), while that of the 3-week-old Milan hypertensive rats (before the main rise in arterial pressure) and their normotensive controls was 99.9 ± 27.4 and 7.8 ± 1.8 U/ml (n = 7; p<0.001). With the use of a semiquantitative cytochemical assay that measures Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity directly, plasma from the adult hypertensive rats had a much greater capacity to inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase than the plasma of the control rats. The significantly raised levels found in the young hypertensive rats before the main rise in arterial pressure are consistent with the hypothesis that the rise in the ability of plasma to inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase is due to an inherited renal difficulty in excreting sodium. (Hypertension 9:498–503, 1987)

KEY WORDS • plasma • glucose-6-phosphate dehydrogenase-stimulating activity • hypertensive rats • cytochemical assay • Na\textsuperscript{+},K\textsuperscript{+}-ATPase-inhibiting activity

WITH the use of cytochemical techniques\textsuperscript{1,2} we have previously demonstrated that plasma from normal humans\textsuperscript{3} and rats\textsuperscript{4} on a high sodium intake, from patients with essential hypertension,\textsuperscript{3} and from adult spontaneously hypertensive rats (SHR)\textsuperscript{6} have a heightened ability to inhibit Na\textsuperscript{+},K\textsuperscript{+}-adenosine triphosphatase (ATPase). It therefore appears that these two forms of hereditary hypertension are associated with a rise in the concentration of a cytochemically bioassayable circulating Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor that is controlled by salt intake in normal humans and animals. In the following experiments the plasma of Milan hypertensive strain rats (MHS) was studied at 3 and 9 weeks of age (i.e., before and after the rise in blood pressure). Much evidence suggests that the cause of hypertension in this strain is a genetically determined renal abnormality\textsuperscript{7} and that, during the development of hypertension, there is a transient period of detectable sodium retention.\textsuperscript{8} These rats may therefore be particularly suitable for testing the hypothesis that a circulating Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor, the concentration of which is known to rise with a high salt intake, may be involved in the pathogenesis of genetic hypertension.

Two cytochemical techniques\textsuperscript{9} were used. One is a validated cytochemical bioassay that measures the capacity of biological samples to stimulate glucose-6-phosphate dehydrogenase (G6PD) activity of fresh guinea pig kidney after exposure for 2 minutes, which is an indication, using the other cytochemical technique, of their capacity to inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase after exposure for 4 to 6 minutes.\textsuperscript{2} The technique that measures Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity is direct but semiquantitative.\textsuperscript{1}

From the Research Laboratories (J. Millett, H. de Wardener) and Department of Chemical Pathology (S. Holland, J. Alaghband-Zadeh), Charing Cross and Westminster Medical School, London, England, and Centro Ricerche Farmitalia (P. Ferrari), Milan, and Instituto di Scienze Mediche (G. Bianchi), dell'Universita di Milano, Milan, Italy.

Supported by a grant from the National Kidney Research Fund and the Fondazione Clarisse Neiman.

Address for reprints: Professor H. E. de Wardener, Research Laboratories, Charing Cross & Westminster Medical School, Fulham Palace Road, London W6 8RF, England.

Received May 7, 1986; accepted November 3, 1986.

498
Materials and Methods

Two groups of 3-week-old rats, one comprising MHS and the other Milan normotensive strain rats (MNS), 10, 11 and two groups of 9-week-old adult MHS and MNS were studied. There were seven rats in each group. Blood was collected in Milan in heparinized test tubes and centrifuged, and aliquots of plasma were then packed in solid CO₂ and transported to London, where 6 hours later they were stored at −20°C. The average sodium and potassium content of the diet for the adult rats was 2.0 and 7.3 g/kg, respectively. The young rats were weaned the day before the blood was obtained.

Cytochemical Techniques

Cytochemical procedures, including the validation of the G6PD assay, have been described elsewhere. 1, 2, 9 A brief summary of the techniques is given here.

Female guinea pigs of the Duncan-Hartley strain (Porcellus Firgrove Farm, Cross-in-Hand, Heathfield, Sussex, England) weighing 300 to 450 g were killed by cervical fracture. Both kidneys were removed, cut into segments, and placed in sealed pots in a nonproliferative culture medium under an atmosphere of 95% O₂, 5% CO₂ at 37°C for 5 hours. The medium was then replaced with fresh medium containing various dilutions of plasma for 2 to 6 minutes (depending on which of the two assays was being used). The segments were chilled by immersion in n-hexane (−70°C), and sections, 16 μm thick, were cut from each segment using a cryostat microtome at −30°C. To demonstrate G6PD activity, the sections were incubated at 37°C under an atmosphere of nitrogen in a reaction medium containing glucose-6-phosphate (5 mM), the coenzyme nicotinamide adenine dinucleotide phosphate (3 mM), neotetrazolium chloride (5 mM), potassium cyanide (10 mM), phenazine methosulfate (0.67 mM), and polyvinyl alcohol (G18 12% wt/vol) dissolved in a 0.05 M glycylglycine sodium hydroxide buffer (pH 8.2). The reaction was stopped by immersing the sections in distilled water. The activity of the G6PD was shown by the deposition of an intensely colored, highly localized formazan. A plasma of high potency was used as a standard. In established cytochemical bioassays for known hormones, the quantitative effect induced by a pure hormone, when measured at a predetermined time of exposure, is only evident over a specific range of low concentrations of the hormone. Similarly, with increasing dilutions of the high potency plasma, stimulation of G6PD measured at the predetermined time of 2 minutes first becomes evident and then reaches a peak of activity before returning to baseline in a log linear relationship (Figure 1). The amount of G6PD-stimulating activity that produces maximum G6PD stimulation at 2 minutes is defined as 1 unit of G6PD activity. The biphasic phenomenon of the dose response is seen in all established cytochemical assays of known hormones. 4 It is attributed to a more rapid effect of the high concentration of hormone on enzyme activity, so that the induced effect on enzyme at such concentrations, after 2 minutes of exposure in this instance, is in the recovery phase and thus appears to be less than that seen at a lower concentration.

To demonstrate Na⁺,K⁺-ATPase activity, 1 the sections from tissue segments were preincubated at 37°C in a medium containing 40% (wt/vol) polypeptide (Polypep 5115; Sigma Chemical, St. Louis, MO, USA) and 0.1 M potassium acetate dissolved in 0.2 M Tris at pH 7.4 for 2 minutes, to remove free phosphate. The preincubation buffer was then replaced for 15 minutes by reaction medium containing 40% (wt/vol) polypeptide, potassium chloride (37.5 mM), sodium chloride (410 mM), magnesium chloride (20 mM), ATP (10 mM), sodium acetate (2 mM), together with lead ammonium citrate/acetate complex (32 g/L; previously dissolved by shaking in the smallest amounts of dilute ammonia) and dissolved in 0.2 M Tris buffer, pH 7.4. The sections were rinsed several times in 0.2 M Tris buffer, pH 7.4, and immersed in water saturated with H₂S for 2 minutes. This procedure demonstrates the total ATPase activity as a brown precipitate. The amount of Na⁺,K⁺-ATPase (i.e., ouabain-sensitive ATPase) was determined by including ouabain (2 × 10⁻⁴ M) in the reaction medium in serial sections, then subtracting the ouabain-insensitive ATPase activity from the total ATPase activity.

Measurement of Glucose-6-Phosphate Dehydrogenase and Na⁺,K⁺-ATPase Activity

The colored reaction products of the enzymes in the proximal tubule were measured using a Vickers MBS scanning and integrating microdensitometer (Vickers Instruments, York, England) at a wavelength of 585 nm and a magnification of ×400, in 20 proximal tubules from duplicate sections from each segment.

![Figure 1. Changes in glucose-6-phosphate dehydrogenase (G6PD) activity, as shown by the amount of formazan deposit measured by microdensitometry (expressed as mean integrated extinction × 100), in proximal convoluted tubules in segments of guinea pig kidney exposed to dilutions of a high potency plasma and plasma from MHS and MNS.](https://hyper.ahajournals.org/content/499/8/1163)
TABLE 1. Weight and Blood Pressure on Day of Experiment

<table>
<thead>
<tr>
<th>Variable</th>
<th>3-week-old</th>
<th></th>
<th>9-week-old</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MNS</td>
<td>MHS</td>
<td>MNS</td>
<td>MHS</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>48.9±7.1</td>
<td>56.4±1.1</td>
<td>273.3±4.2</td>
<td>293.1±9.2</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
<td>117.5±1.3</td>
<td>124.3±1.8*</td>
<td>133.6±1.0</td>
<td>172±1.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE.

*p < 0.005, †p < 0.001, compared with respective value in MNS.

Results

The weight and systolic blood pressure, measured at the tail (W + W blood pressure recorder; Basile, Italy), are given in Table 1. To avoid disturbing the weanling rats, on the day that blood was obtained, the blood pressure measurements were made on another group of rats of the same age.

Adult Rats

The mean G6PD-stimulating capacity of the hypertensive strain rat plasma was 586 ± 88.8 U/ml and of the normotensive strain plasma was 23.4 ± 8.3 U/ml (p < 0.001; Figure 2). Figure 3 illustrates the time course of the ability of plasma from adult MHS and MNS to inhibit Na⁺,K⁺-ATPase directly at 2, 4, 6, and 8 minutes and demonstrates that the plasma of MHS had a considerably greater capacity to inhibit Na⁺,K⁺-ATPase than the plasma of the control rats.

Young Rats

The mean G6PD-stimulating activity of the hypertensive strain rat plasma was 99.9 ± 27.4 U/ml and of the normotensive strain rat plasma was 7.8 ± 1.8 U/ml (p < 0.01).

Statistical Validation

The mean index of precision for the individual assay results was 0.1389 (n = 28), with fiducial limits (p < 0.95) ranging from 84–119% to 13–143% (n = 28). There was no statistical divergence from parallelism between the log-dose response of the human plasma used as a standard and 27 of the 28 plasma samples assayed. Values are reported as means ± SE.

Discussion

In the present study, cytochemical techniques were used to assay the capacity of diluted whole plasma to inhibit Na⁺,K⁺-ATPase activity and to stimulate G6PD activity in guinea pig proximal tubule. Cytochemical bioassays of hormones are based on the prin-
ciple that when a hormone binds to its receptor on a living cell it produces a transient, rapidly reversible effect on an enzyme that can be revealed by means of a colored end product at the site of enzymatic activity. 

Ouabain, normal human plasma, and rat plasma induce a reversible inhibition of Na⁺,K⁺-ATPase in proximal tubules that is maximal at 4 to 6 minutes (Figure 4). In cytochemical assays of known hormones the time at which the enzymatic change is maximal is one of the features that confers specificity. With the use of this technique the plasma of a group of salt-loaded men inhibited 25 times more Na⁺,K⁺-ATPase than when they were on a low salt diet. 

The cytochemical technique with which inhibition of Na⁺,K⁺-ATPase activity is measured, however, is semiquantitative in that, although it is possible to obtain some quantitative information from the time course, the small amount of baseline Na⁺,K⁺-ATPase activity remaining in the kidney section makes it difficult to obtain a dose versus inhibiting response of sufficient steepness for a reproducible quantitative assay. Inhibition of Na⁺,K⁺-ATPase in fresh tissue, however, is associated with stimulation of G6PD activity, and the dose-response curve, with dilutions of plasma, is linear and of sufficient steepness to validate a quantitative assay that measures the capacity of body fluids and extracts to stimulate G6PD as an index of their ability to inhibit Na⁺,K⁺-ATPase. The validation criteria include a statistical evaluation of the index of precision (λ), parallelism with the standard, and fiducial limits for each assay. 

Ouabain and normal human and rat plasma induce a reversible rise in G6PD activity that is maximal at 2 minutes (see Figure 4). The plasma of salt-loaded humans and rats stimulates G6PD about 20 times more than when these species are deprived of sodium. Tests of selectivity have been performed on various substances, many of which are known to have metabolic effects on the proximal tubule and some of which have been demonstrated to inhibit purified preparations of Na⁺,K⁺-ATPase, including fatty acids (linoleic and oleic), dehydroepiandrosterone sulfate, lyso-phosphatidyl choline, and sodium vanadate. None stimulated G6PD at 2 minutes. Therefore, the cytochemical techniques that detect the presence of a substance that inhibits Na⁺,K⁺-ATPase at 4 to 6 minutes and stimulates G6PD at 2 minutes have a certain selectivity. The effect of ouabain is only evident at relatively high concentrations (10⁻² M to 10⁻⁶ M), which suggests that the affinity of the endogenous Na⁺,K⁺-ATPase inhibitor for the tubule cell receptor is higher than that of ouabain itself. 

The results obtained with these cytochemical techniques show that at 9 weeks, when hypertension is fully developed, the capacity of the plasma of MHS to inhibit Na⁺,K⁺-ATPase activity at 4 to 6 minutes and to stimulate G6PD activity at 2 minutes is considerably greater than that of the plasma of MNS. This finding supports the notion that an endogenous inhibitor of Na⁺,K⁺-ATPase activity is present in greater amounts in plasma of MHS, and it is in keeping with the observation that the ouabain-sensitive Na⁺ efflux from fresh erythrocytes is lower in the adult MHS than in the MNS. 

At 3 weeks the plasma of MHS has a significantly greater capacity to stimulate G6PD than that from the MNS, although the difference is much lower than it is at 9 weeks. At 3 weeks the ouabain-sensitive Na⁺ efflux from fresh erythrocytes is similar in both strains. The blood pressure of the group of 3-week-old rats on which this measurement was made was slightly but significantly raised. But, as discussed elsewhere, blood pressure differences, when measured on different occasions, between the hypertensive and the normotensive strains at 3 weeks of age is not always statistically significant. 

The finding that the capacity of the plasma to stimulate G6PD activity is raised at 3 weeks in the MHS, before the main rise in arterial pressure has occurred, supports the hypothesis that the rise in the capacity of the plasma to stimulate G6PD activity and to inhibit Na⁺,K⁺-ATPase activity in the adult rat is not secondary to the rise in arterial pressure per se, or to a secondary effect of the hypertension on renal function. The raised plasma capacity to stimulate G6PD in the MHS probably is due to an intensified need to increase sodium excretion because of an inherited defect in the ability of the kidney to excrete sodium. There is some experimental evidence that supports the existence of such a defect in the MHS. Renal cross-transplantation experiments between MHS and MNS have demonstrated that the arterial pressure of the recipient is determined by the kidney it receives. The normotensive strain rat becomes hypertensive, while the hypertensive strain rat’s blood pressure decreases or does not rise, which demonstrates that the hypertension is due to some genetic fault in the kidney of the MHS. Many individual differences in renal function and sodium 

**Figure 4.** Percentage changes in glucose-6-phosphate dehydrogenase (G6PD) activity (○) and Na⁺,K⁺-ATPase activity (●) in proximal tubules of segments of fresh guinea pig kidney exposed to 1:1000 plasma and 4 × 10⁻⁴ M ouabain for 2 to 8 minutes.
balance between the MHS and the MNS are detectable before the development of hypertension. Glomerular filtration rate, urinary output, and renal excretion of a sodium load are greater in the MHS,8,12,23 while basal renal sodium output is equal.8 Perfused isolated kidneys from young MHS show increased tubular sodium reabsorption and O$_2$ consumption,24 and isolated membranes of proximal tubule cells from the kidney of MHS have an increased sodium transport.25 Presumably these differences in sodium reabsorption give rise to the transient cumulative sodium retention observed in the MHS during the development of hypertension.8 Evidence for increased proximal tubule sodium reabsorption has also been obtained in patients with essential hypertension and in the SHR.26,27

The high degree of selectivity that the cytochemical assays confer appears to be for a substance that is normally controlled by changes in salt intake and fluid volume.4,9 The only connection that has so far emerged with the substance in the plasma that inhibits Na$^+$.K$^+$.ATPase at 4 to 6 minutes and stimulates G6PD at 2 minutes using cytochemical techniques is ouabain.

This work demonstrates that, using cytochemical techniques, the ability of the plasma of MHS to stimulate G6PD and to inhibit Na$^+$.K$^+$.ATPase is concomitant with the development of hypertension, which suggests that a substance with ouabainlike biocactivity may be involved in the rise of arterial pressure. An increase in the plasma's cytochemically detectable Na$^+$.K$^+$.ATPase-inhibiting and G6PD-stimulating activity has also been demonstrated in established essential hypertension, in adult SHR, and in normal subjects and rats on a high sodium intake. It is possible that in these three forms of hypertension the increase in the cytochemically detectable circulating Na$^+$.K$^+$.ATPase inhibitor is due to the observed, acquired increase in intrathoracic pressure.28,29 which is due in turn to a genetic increase in proximal tubule sodium reabsorption.26,27

References
7. Birkenhager WH, Reil JL, eds. Handbook of hypertension; vol 4}

Downloaded from http://hyper.ahajournals.org/ by guest on November 12, 2017
Cytochemically assayable Na+,K+-ATPase inhibition by Milan hypertensive rat plasma.
S Holland, J Millett, J Alagband-Zadeh, H de Wardener, P Ferrari and G Bianchi

Hypertension. 1987;9:498-503
do: 10.1161/01.HYP.9.5.498

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1987 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/9/5/498

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/