Endogenous Digitalislike Circulating Substances in Spontaneously Hypertensive Rats

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SUMMARY Circulating digitalislike compounds have been proposed to be involved in some Na+-dependent types of experimental hypertension and in human essential hypertension. The level of circulating Na+-K+ pump inhibitor(s) was investigated in the spontaneously hypertensive rat of the Okamoto strain (SHR), its normotensive control, Wistar-Kyoto rat (WKY), and the regular Wistar rat using the following criteria: the ability of whole plasma to inhibit the total active Na+ efflux from Wistar rat erythrocytes and to cross-react with digoxin antibodies and the ability of plasma extracts to inhibit Na+,K+-adenosine triphosphatase (ATPase) activity of membranes from rat kidney. SHR plasma inhibited the net Na+ efflux from Wistar erythrocytes by up to 27% compared with WKY or Wistar plasma. For a given number of cells, the inhibition increased with the amount of available plasma. Cross-reactivity with digoxin antibodies was twice as high in SHR as in WKY or Wistar plasma. It was already enhanced in 3- to 4-week-old rats. Plasma extracts from SHR significantly inhibited Na+,K+-ATPase activity when compared with WKY extracts (75.6 ± 2.6 vs 89.3 ± 2.4 µmol Pi/mg/hr; p < 0.01) but did not differ from Wistar plasma extracts. These results strongly suggest that circulating digitalislike compound(s) are present in elevated amounts in SHR as early as 3 to 4 weeks of age, but their exact participation in blood pressure elevation or maintenance remains to be clarified.

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KEY WORDS • spontaneously hypertensive rats • Wistar-Kyoto rats • endogenous Na+-K+ pump inhibitors • Na+,K+-adenosine triphosphatase • digoxin radioimmunoassay • sodium fluxes

ENDOGENOUS digitalislike compound(s) have been proposed to participate in Na+ and water homeostasis and in the development of hypertension by inhibiting the ouabain-sensitive Na+-K+ pump.1-3 De Wardener and MacGregor3 suggested that, in the presence of an intrinsic or acquired defect in renal Na+ excretion, such inhibitors of active Na+ transport would be released. At the renal level, they would decrease the tubular Na+ reabsorption and increase Na+ and water excretion. At the vascular smooth muscle level, they are thought to increase intracellular Na+ and Ca2+ concentrations, basal tone, and contractile response.1-3 An enhanced level of these inhibitors might thus participate in a rise in blood pressure. Several investigators have reported an increased activity of these ouabainlike substances in the plasma and urine of patients with essential hypertension,4-7 as well as in acute volume expansion8-9 and in some types of experimental hypertension.10-15 Their presence in the spontaneously hypertensive rat of the Okamoto strain (SHR) is still a matter of controversy.

The early studies of Yamori16 on the effects of parabiosis between SHR and Wistar-Kyoto rats (WKY) failed to provide evidence for a circulating hypertensive substance in SHR. According to a few reports, however, SHR plasma contains some unidentified substance(s) able to modify the characteristics of vascular smooth muscle. Greenberg et al.17 demonstrated that the blood pressure of WKY in parabiosis with SHR increased slowly during the first 4 weeks of parabiotic union with SHR, then rapidly over the next 3 months, to reach the same pressure levels as those of the SHR strain. They observed concomitant and progressive changes in WKY venous contractility, extensibility, and hypertrophy that also reached levels seen in SHR. Wright reported that plasma from SHR enhanced the respon-
siveness of arterial strips to norepinephrine more than did plasma from WKY or Sprague-Dawley rats and that SHR blood contains a substance that produces an elevation in blood pressure in normotensive rats. The observation by Murtry et al. that normotensive rat pups raised by SHR mothers had a sustained elevation in their blood pressure compared with pups left with their natural mothers has been interpreted as a consequence of either the hyperkinetic behavior of the mother or the transmission by the mother's milk of a substance that increases systolic blood pressure in the young normotensive rat. The presence of high amounts of compound(s) able to inhibit the active Na+ transport in plasma and hypothalamus of SHR has been recently reported on the basis of indirect or semi-quantitative cytochemical assays. In contrast, in vitro experiments on cell Na+ transport did not confirm that active Na+ transport inhibitors are present in SHR plasma. Studies of plasma-induced changes in Na+ fluxes from erythrocytes, white blood cells, or rat thymocytes favor the participation of an inherited membrane defect in SHR, rather than the participation of a plasma substance.

The goal of the present work was to test the hypothesis of enhanced levels of circulating digitalislike compounds in SHR when compared with their age-matched normotensive WKY controls and with regular Wistar rats. The digitalislike activities were evaluated by three tests: 1) the ability of whole plasma to inhibit total Na+ efflux from Wistar rat erythrocytes, 2) the ability of whole plasma to cross-react with antidigoxin antibodies, and 3) the ability of plasma extracts to inhibit the Na+,-K+-ATPase activity of isolated membranes from rat kidney. Intraerythrocyte Na+ content was also measured as a partial reflection of an in vivo inhibition of active Na+ transport. Plasma nonesterified fatty acids (NEFAs) were quantified, as some have been reported to exhibit digitalislike activity.

Materials and Methods

Rats

Age and sex–matched 3- to 30-week-old male SHR, normotensive WKY, and regular Wistar controls, derived from the National Institutes of Health stock, were purchased from Iffa Credo (Les Oncins, France). They received a standard diet (0.2% NaCl) and tap water ad libitum.

Preparation of Plasma Samples

Arterial blood was withdrawn from the carotid artery of anesthetized rats (pentobarbital, 45 mg/kg), collected in a chilled tube containing Na+ heparin (50 U/ml, Choay Biosys, Compiègne, France), and centrifuged at 2500 g for 5 minutes at 4 °C.

Digitalislike properties were studied on whole or deproteinized plasma, either immediately or after storage at −80 °C. Plasma extracts were immediately prepared as follows: plasma was boiled in a water bath for 15 minutes; the protein network was then disrupted and centrifuged at 50,000 g for 30 minutes at 4 °C. The supernatant was kept at −80 °C until analysis, either as such or lyophilized and taken up in the same volume as the initial plasma volume just before analysis.

Effect of Plasma on Net Sodium Extrusion from Rat Erythrocytes

Adult Wistar rat arterial blood was obtained as described, and red blood cells free of buffy coat were washed three times in an isotonic NaCl solution by centrifugation at 3000 g for 2 minutes at 4 °C. One milliliter of the final erythrocyte pellet was added to 40 ml of a Na+ loading solution containing 155 mM NaCl, 1 mM MgCl₂, 2.5 mM sodium phosphate buffer, pH 7.4. The suspension was placed in a shaking water bath at 37 °C for 2 to 4 hours to enhance progressively the Na+ concentration up to 16 mmol/L of cells.

During that time, blood was withdrawn from anesthetized SHR, WKY, and Wistar rats and plasma was prepared as described. At the end of the Na+ loading period, red blood cells were diluted to a 1 to 5% hematocrit in 0.5 ml of plasma to be studied. In a first set of experiments, hematocrit was kept constant at 2% while internal erythrocyte Na+ concentration was enhanced up to 16 mmol/L of cells. In a second set of experiments, hematocrit was varied while initial internal erythrocyte Na+ content was maintained between 12.5 and 13.5 mmol/L of cells. After 1 hour at 37 °C, the reaction was stopped by adding 3 ml of ice-cold isotonic NaCl solution. The cells were washed by centrifugation (3000 g, 1 minute, three times) in an ice-cold solution containing 112 mM MgCl₂ and 0.1 mM ouabain and then lysed by adding 4 ml of distilled water. Ghosts were removed by a 50-minute centrifugation at 6000 g. Intracellular Na+ concentration was determined by flame photometry and hemoglobin by spectrophotometry. The difference between intraerythrocytic Na+ content before and after incubation in plasma was considered to be the net Na+ extrusion.

Cross-reactivity with Antidigoxin Antibodies

The digoxinlike immunoreactivity was measured on whole plasma according to the method previously described. Plasma from 3- to 30-week-old rats was tested for levels of digoxin using a commercial radioimmunoassay kit (New England Nuclear, Dupont, Paris, France). The sensitivity of the assay was increased by incubating 100 μl of plasma with 50 μl of antidigoxin serum solution and 50 μl of [125]I-digoxin tracer. Bound antigen was separated from free antigen by differential absorption on activated charcoal for 10 minutes. Aliquots of supernatant were removed and counted. Fifty percent inhibition was obtained with digoxin, 217 ± 9 pg/ml (n = 23), and digoxin concentra-
Inhibition of Rat Kidney Na\(^{+}\),K\(^{+}\)-ATPase Activity

Na\(^{+}\),K\(^{+}\)-ATPase-enriched membranes from rat kidney were prepared as described previously. Kidneys from male Wistar rats were perfused with ice-cold heparinized isotonic NaCl. The cortex and medulla were dissected away from the papilla and connective tissue and homogenized in a glass-Eveljhem potter in 10 ml/g tissue of a medium containing 1 mM MgCl\(_2\), 250 mM sucrose, 1 mM EDTA, 10 mM Tris HCl, pH 7.4. Membranes were obtained by centrifuging twice at 800 g for 15 minutes at 4 °C followed by two centrifugations at 5000 g for 30 minutes at 4 °C. The final pellet was taken up in 1 mM EDTA, 58 mM Tris buffer, pH 7.4. Na\(^{+}\),K\(^{+}\)-ATPase-enriched membranes were incubated with sodium dodecylsulfate, 0.3 mg/mg protein (1 mg protein/ml suspension) for 20 minutes at room temperature, then washed in 10 ml of 1 mM EDTA, 58 mM Tris buffer, pH 7.4, by centrifugation for 30 minutes at 50,000 g and stored at 2 mg/ml in aliquots at -80 °C. For measurement of total ATPase activity, the incubation medium (100 y) contained 2 fig of Na\(^{+}\),K\(^{+}\)-ATPase-enriched membranes, 10 to 30 nCi \[^{32}\text{P}\]adenosine 5'-triphosphate (ATP; Amersham France, Les Ulis, France), 2 mM Na-ATP, 3 mM EGTA, 2 mM KCl, 80 mM HEPES, pH 7.4, and a two-fold concentration of plasma extracts from 20- to 25-week-old experimental or control rats. The final ionic concentration averaged 280 mM Na\(^{+}\), 10 mM K\(^{+}\), 2 mM Ca\(^{2+}\), and 5 mM Mg\(^{2+}\). This concentration allowed the enzyme to work under conditions of maximal velocity. Ouabain-insensitive ATP hydrolysis was measured in a similar medium without K\(^{+}\) and in the presence of 1 mM ouabain. The reaction was stopped by refrigeration at 0 °C and the addition of 100 µl of 23% perchloric acid.

Measurement of Plasma Nonesterified Fatty Acids

NEFAs were measured in whole plasma and plasma extracts by an enzymatic method (NEFA C test WAKO) using a centrifugal analyzer (Cobas Bio, Roche, Neuilly, France).

Statistical Analysis

The null hypothesis was tested using analysis of variance and the two-tailed unpaired Student's t test when required. Correlation coefficients were calculated from linear regression by the least-squares method. Data are expressed as means ± SEM.

Results

Characteristics of the Rats

Systolic blood pressure, hematocrit, intraerythrocytic Na\(^{+}\) content, and plasma NEFAs were measured at different ages, as indicated in Table 1. SHR hematocrit was the same as that of WKY.
(except at 10-12 weeks of age) and higher than that of Wistar rats. At all ages considered, erythrocyte Na$^+$ content from SHR was higher than that of WKY and, except at 3 to 4 weeks of age, was similar to that of Wistar rats. NEFAs in the three strains were measured in whole plasma and after deproteinization by boiling. Boiling induced a nearly total loss of NEFAs in SHR and WKY plasma. This result renders unlikely any participation of NEFAs in the digitalislike activity of plasma extracts.

**Effect of Plasma on the Net Na$^+$ Extrusion from Wistar Rat Erythrocytes**

Na$^+$-loaded erythrocytes from Wistar rats were distributed in plasma from adult rats of the three strains, and the Na$^+$ fluxes were measured in the absence and presence of $10^{-3}$ M ouabain to evaluate the activity of circulating inhibitor(s) of active Na$^+$ transport. With all three plasma samples, net Na$^+$ efflux was always higher than that observed in the presence of plasma from Wistar rats or WKY. For instance, at the maximal internal Na$^+$ concentration studied (i.e., 16 mmol/L of cells), a 27% inhibition was obtained with the SHR plasma when compared with that observed in WKY or Wistar rat plasma. This inhibition could not be explained by differences in the ionic Na$^+$ or K$^+$ plasma content, which were similar in the three strains, and was thus considered to reflect the presence of a Na$^+$ transport inhibitor.

The ouabain-insensitive Na$^+$ fluxes measured in the presence of $10^{-3}$ M ouabain and plasma from the three strains were similar. After 1 hour, they reached 2.59 ± 0.56, 2.37 ± 0.15, and 2.63 ± 0.71 mmol/L of cells in the presence of plasma from SHR, WKY, and Wistar rats, respectively. The lowered net Na$^+$ fluxes observed in the presence of SHR plasma thus indicate a decreased Na$^+$-K$^+$ pump activity.

To better evaluate the activity of circulating digitalislike compounds, the net Na$^+$ fluxes were measured under various conditions. Based on the assumption that the available concentration of these putative Na$^+$ transport inhibitor(s) per cell would determine the level of inhibition, the measurements were performed in plasma from the three strains with hematocrits ranging from 0.5 to 4.5%. For a constant amount of plasma, the lower the number of target cells, the greater the inhibition of Na$^+$ efflux from target erythrocytes (Figure 2). At four of the five hematocrits used, plasma from SHR inhibited the net Na$^+$ effluxes more than did plasma from WKY and Wistar rats. Beyond a ratio of 1% cells/ml plasma, outward Na$^+$ fluxes from the cells were no longer observed in the presence of plasma from SHR. Only a net Na$^+$ influx down the transmembrane Na$^+$ gradient was measured (2.88 ± 0.31 mmol/L of cells). This finding was similar to the net Na$^+$ influx observed in erythrocytes in the presence of plasma and $10^{-3}$ M ouabain.

**Apparent Immunoreactivity of Plasma Obtained from SHR, WKY, or Wistar Rats**

Plasma from the three strains cross-reacted with digoxin antibodies, and the apparent immunoreactivity increased with age for SHR and WKY (Figure 3). Regular control Wistar rat plasma contained the same amount of digoxin equivalents regardless of age. Plasma from SHR had a 1.6-fold to 2.7-fold greater concentration of immunoreactive material compared with that of age-matched WKY and Wistar rats. Furthermore, the digitalislike crossreactivity was already twice as high in the plasma from 3-week-old SHR as in that from age-matched WKY, prior to a significant rise in blood pressure (103 ± 4 vs 39 ± 3 pg/ml; n = 21 and 22, respectively, p < 0.001).

**Na$^+$,K$^+$-ATPase Activity in the Presence of Plasma Extracts from SHR, WKY, or Wistar Rats**

The enzymatic activity of a preparation of rat kidney membranes enriched in Na$^+$,K$^+$-ATPase was
measured in the presence of plasma extracts from adult SHR, WKY, or Wistar rats. When compared with plasma extracts from WKY, those from SHR inhibited the Na\(^+\),K\(^+\)-ATPase activity (75.6 ± 2.6 vs 89.3 ± 2.4 µmol P\(_i\)/mg/hr; n = 11 and 10, respectively, p < 0.01; Figure 4). However, the enzymatic activity in the presence of plasma extracts from Wistar rats did not significantly differ from that obtained with SHR plasma (81.5 ± 2.5 vs 75.6 ± 2.6 µmol P\(_i\)/mg/hr; n = 13 and 11, respectively).

**Relationships Between Plasma Digitalislike Properties and Biological Parameters**

To investigate whether the plasma digitalislike compounds have any physiological effects on cell Na\(^+\) handling or the rise in blood pressure, their relationships with erythrocyte Na\(^+\) content and blood pressure were analyzed. Individual values of the apparent digoxinlike immunoreactivity and of the ability of plasma extracts to inhibit renal Na\(^+\),K\(^+\)-ATPase, measured in adult SHR and WKY, were correlated (r = -0.710, n = 14, p < 0.01; Figure 5A). When individual values of Wistar rats were added, this relationship was at the limit of significance (r = -0.430, p = 0.05, n = 21).

When individual values for SHR, WKY, and Wistar rats were pooled as a function of their age, mean values of systolic blood pressure and apparent digoxinlike immunoreactivity were correlated (r = 0.822, n = 12, p < 0.01; Figure 5B), but there was no correlation between the mean apparent digoxinlike immunoreactivity and the mean erythrocyte Na\(^+\) content (r = 0.279, n = 12). A significant correlation was found between these two parameters when only SHR and WKY were considered (r = 0.816, n = 8, p < 0.02; Figure 5C).

**Discussion**

Circulating digitalislike compounds are reportedly present in elevated amounts in volume expansion,\(^1\) in some types of experimental hypertension,\(^1\) and in human essential hypertension.\(^3\) The purpose of this study was to investigate whether such compounds are present in the SHR, an animal model exhibiting some characteristics similar to those present in essential hypertension.\(^32\),\(^33\)

We found that, compared with that of both WKY and Wistar rats, SHR plasma was characterized by an enhanced inhibitory effect on active Na\(^+\) efflux from red blood cells and a higher cross-reactivity with antidigoxin antibodies that was present before a rise in blood pressure. When compared with that of WKY and Wistar rats, plasma from SHR signifi-
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...with that in the presence of Wistar or WKY plasma. As the SHR and WKY ouabain-insensitive fluxes were similar, the lowered net Na⁺ efflux in SHR plasma reflected a lowered activity of the Na⁺-K⁺ pump. The best conditions for observing inhibition of active Na⁺ efflux from cells clearly occur when the turnover of Na⁺-K⁺ pump is high (i.e., when it is activated by elevated amounts of internal substrate).

This prerequisite might explain why Van de Ven and Bohr23 or Feig et al.,24 who did not raise the internal Na⁺ content of target cells, did not observe differences in the net Na⁺ effluxes from red blood cells incubated in SHR or control plasma and concluded that the participation of membrane defects was more likely than that of circulating substance(s). This condition also implies that the circulating inhibitor(s) would be physiologically efficient only in cells with a high Na⁺ content, such as smooth muscle cells, and not in erythrocytes, in which the internal Na⁺ concentration is particularly low.

Plasma from SHR also contained higher amounts of compound(s) able to cross-react with antidigoxin antibodies than did those of both WKY and Wistar rats. Their apparent immunoreactivity was already markedly enhanced before any significant rise in blood pressure had occurred. The specificity of the radioimmunoassay used argues against a participation of the major circulating steroids, as, among the 25 steroid compounds tested, those having the highest cross-reactivity were progesterone and hydrocortisone derivatives, which require a concentration in the 10⁻⁵ M range to cause a 50% displacement.31 The increased digoxinlike immunoreactivity observed in SHR plasma contrasts with...
that measured in urine by Yasuhara et al., who reported similar amounts of digitalislike substances in urinary extracts from WKY and SHR, whether prehypertensive or adult. Such a discrepancy suggests either that the specificities of their and our antibodies differ or that the circulating immunoreactive compound(s) may have been metabolized into urinary nonreactive material. To date, there is no evidence that plasma and urine contain the same immunoreactive substance.

Another criterion used to establish the digitalislike properties of plasma is the inhibition of Na+,K+-ATPase activity. It gave a less clear-cut response: Whereas the enzymatic activity of renal membranes was lower in the presence of plasma extracts from SHR than in those of WKY and Wistar rats, only the difference with WKY reached significance. This observation and the finding that the inhibition was rather small (15.3%) call for two comments. One concerns the heterogeneity of renal Na+,K+-ATPase. Doucet and Barlet showed that the affinity of ouabain for renal Na+,K+-ATPase increased up to 30 times from the proximal to the distal part of the rabbit nephron. This increase implies structural changes of the enzyme itself or of its environment and preferential inhibition of the enzymes with the highest affinities for ouabain by low concentrations of ouabain or related compounds. A weak overall inhibitory effect might thus reflect a marked inhibition in a few sites of the nephron without changes in the others.

A second point is the use of appropriate normotensive controls for SHR. As recently pointed out by Rapp and Kurtz and Morris, the WKY strain is far from an ideal control for SHR. Their biological variability and the possible selection of physiological or biological traits unrelated to high blood pressure demand caution in interpreting the differences between SHR and WKY. For example, the lower erythrocyte Na+ content in WKY when compared to SHR and Wistar rats may suggest that it is the WKY red blood cells that are abnormal. The higher Na+,K+-ATPase activity of renal membranes in the presence of WKY plasma extracts may reflect a similar abnormality.

It is difficult to reconcile these ambiguous results concerning the Na+,K+-ATPase inhibition with those of Millet et al., who reported that, compared with that of WKY, plasma from SHR exerts a very marked stimulation of the activity of the renal glucose 6-phosphate dehydrogenase in fresh guinea pig kidney proximal tubule; this stimulation was usually associated with reciprocal changes in Na+,K+-ATPase activity. They have also confirmed that SHR plasma does induce a transient inhibition of Na+,K+-ATPase activity in the same cells. The difference between these and our results cannot be explained by different Na+ intakes, as with the diets of both their and our animals the urinary Na+ excretion averaged 1 mmol/24 hr. Methodological differences are more likely to be involved, including the origin of the Na+,K+-ATPase (rat or guinea pig semipurified enzyme or tissue slices), the amount of plasma studied (1.8 or down to 10⁻⁵), the time course of inhibition (stable or transient), and our use of deproteinized plasma, not whole plasma such as Millet et al. used, because of the high nonspecific inhibition observed with whole plasma under our experimental conditions. The results of these authors are, however, in agreement with the increased inhibition of Na+ fluxes from erythrocytes and the high digoxinlike immunoreactivity of SHR plasma that we described.

Results of measurements of NEFA content in whole or deproteinized plasma from WKY or SHR render unlikely their participation in the inhibition observed, as their total concentration was lower than that reported to inhibit the Na⁺,K⁺ pump.

The present results raise several questions concerning the possible relationships of the plasma digitalislike activity with the expansion of body fluid volumes, which has been proposed as a triggering factor, and the rise in blood pressure, the proposed consequence of their action. Relative Na+ retention has been observed in SHR both before and during the early developmental phase of hypertension. Data concerning the body fluid volumes in young SHR remain conflicting: Trippodo et al. and Rippe et al. have reported that 3- to 6-week-old SHR have a plasma volume, blood volume, and extracellular fluid volume similar to those in age-matched WKY controls. However, plasma volume was observed to be elevated and extracellular fluid volume to be lowered in 4-week-old animals. These results also contrast with the expansion of the extracellular fluid volume and interstitial fluid space without changes in plasma volume observed by Mullins in 12-day-old SHR. These conflicting data may reflect strain variability (as discussed). Methodological differences, or the fleeting nature of the transient changes. Body fluid regulation by these compounds is, however, compatible with the normal or decreased blood and plasma volumes observed in adult SHR by Trippodo et al. and Bianchi et al.

The present results also raise the question of the possible hypertensive role of digitalislike compounds. High amounts of the circulating digitalislike immunoreactive compounds are present in 4-week-old SHR, when the blood pressure is still in the normotensive range. They could therefore participate in the rise of blood pressure. The positive correlation between systolic blood pressure and the plasma digitalislike immunoreactivity observed in this study also agrees with this proposal. A prohypertensive role of these circulating compounds is supported by the antihypertensive effect of the injection of antibodies specifically directed against digitalis compounds observed in several types of experimental hypertension, including genetic hypertension, although the injection in SHR of
Fab fragments of antidigoxin antibodies reportedly does not modify blood pressure. 

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