Vol. 122, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1984 Pages 129-136

CIRCULATING DIGITALIS-LIKE SUBSTANCE IS INCREASED IN DOCA-SALT HYPERTENSION

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Received May 25, 1984

Blood pressure and digitalis-like substance were measured in the plasma of control, salt-treated, and DOCA-salt treated rats. Blood pressure in DOCA-salt treated rats was significantly higher than that of either control or salt-treated animals. Digitalis-like activity was measured by two methods, radioimmunoassay for digoxin, and a receptor binding assay employing a rat brain synaptosomal membrane fraction. Digoxin-like immunoreactivity in plasma was not detected in either control or salt-treated rats, but was detected in DOCA-salt treated rats. Receptor binding activity in salt-treated rats was slightly but significantly higher than that of control rats. In DOCA-salt treated rats, receptor binding activity was significantly higher than that of salt-treated rats. Partial purification of the digitalis-like substance in plasma was performed by gel filtration using Sephadex G-25. Two peaks containing digoxin-like immunoreactivity were observed. Receptor binding activity, as well as Na^+-K^+ ATPase inhibitory activity, was detected only in the second peak, in which approximately 70% of the digoxin-like immunoreactivity was eluted. These results indicate that a circulating digitalis-like substance is increased in DOCA-salt hypertension.

The possible role of a natriuretic substance in the genesis of hypertension was first proposed by Dahl et al. (1). Based on data obtained in parabiosis experiment using salt-sensitive and salt-resistant strains of Dahl's rat, these authors suggested that a sodium-excreting hormone might induce hypertension. Haddy and Overbeck (2) extended this concept and postulated that a circulating sodium pump inhibitor might play a critical role in hypervolemic hypertension. Growing evidence indicates that natriuretic hormone is an endogenous inhibitor of Na^+-K^+ ATPase (3). Of particular interest are recent data showing that it resembles digitalis in terms of both mode of action and immunoreactivity (3). De Wardener and MacGregor (4) proposed that natriuretic hormone might be a factor which causes a sustained

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0006-291X/84 $1.50

rise in blood pressure in various types of hypertension including essential hypertension. This hypothesis is intriguing since it presents an answer to the question how sodium retention might induce hypertension.

Recently, we reported that administration of anti-digoxin antibody to DOCA-salt hypertensive rats resulted in a transient decrease in their blood pressure (5). This result suggests that an endogenous substance which reacts with anti-digoxin antibody is relevant to the maintenance of high blood pressure in DOCA-salt hypertension. In order to evaluate further the role of an endogenous digitalis-like substance in this volume expanded hypertension, I examined circulating digitalis-like substance in DOCA-salt hypertension. Results obtained in this study show clearly that a circulating digitalis-like substance is increased in DOCA-salt hypertension.

Methods

Preparation of DOCA-salt hypertension rats

Male Wistar rats weighing 100-120 g were fed on laboratory rat chow and given deionized distilled water ad lib. After a week they underwent unilateral nephrectomy under ether anesthesia. After this surgery, rats were divided into three groups. In the first group, rats were given 0.9% NaCl solution as drinking water, and each animal was injected twice a week with DOCA suspended in sesame oil at a weekly dosage of 10 mg (DOCA-salt treated group). In the second group, rats were given 0.9% NaCl solution as drinking water and sesame oil alone was injected as vehicle twice a week (salt-treated group). In the third group, rats were given deionized distilled water and sesame oil alone was injected (control group). Systolic blood pressure was measured on unanesthetized warmed rats by the tail cuff method once a week. Six weeks after operation rats were killed by decapitation and blood was collected into heparinized plastic tubes. Plasma was separated by centrifugation and stored at -20°C.

Assay for digitalis-like activity

Digoxin-like immunoreactivity was measured by radioimmunoassay according to the method of Gruber et al. (6) using an anti-digoxin antibody described previously (5). This antibody shows less than 0.1% cross-reactivity with digitoxin and none with other cardiac glycosides nor adrenal steroids.

Binding activity to digitalis receptor was assessed by measuring inhibition of ^{3}H-ouabain binding using rat brain synaptosomal fraction according to the method described by Lichtstein and Samuelov (7). When plasma samples were measured in this assay, 1 ml of plasma was boiled for 5 min to precipitate large protein, and then centrifuged at 8000 x g for 10 min. Supernatant was collected and lyophylized after desalting with a small column of a mixed bed resin (Amberlite MB3). The lyophylizate was reconstituted with 300 µl of 50 mM Tris-HCl (pH 7.4) buffer and applied to the assay. The activity of Na^{+}-K^{+} ATPase was assayed by using brain

synaptosomal fraction (7). Fractions containing digoxin-like immunoreactivity were pooled and lyophylized. After desalting on a small column of mixed bed resin, each fraction was added to an aliquot of the synaptosomal fraction.

Fractionation of pooled plasma

Pooled plasma was boiled for 5 min and then centrifuged for 20 min at 10,000 x g. The supernatant was collected and lyophylized. Samples were fractionated by column chromatography using Sephadex G-25 (7). The size of the Sephadex bed was 1 x 40 cm with a void volume of 18 ml as estimated from the elution of Dextran Blue. The column was eluted with 50 mM Tris-HCl buffer (pH 7.4) at a flow rate of 0.9 ml/min, and 0.5 ml fractions of the eluate were collected.

Treatment with protease

Fractions containing digitalis-like activity were incubated with 50 µg of either trypsin or chymotrypsin for 2 hours at 34°C.

Results

Six weeks after nephrectomy systolic blood pressure and digitalis-like activity in plasma were determined (Table 1). Systolic blood pressure in salt-treated rats was slightly higher than that in control rats, however, this difference was not statistically significant. In DOCA-salt treated rats the mean value ± S.E. of systolic blood pressure was 130.6±7.8 mm Hg which was significantly higher ($p<0.05$) than that in either control (106±6.2 mm Hg) or salt-treated animals (112.1±5.1 mm Hg).

Table 1. Blood Pressure and Digitalis-like Activity in Plasma
Systolic blood pressure and digitalis-like activity were determined as described in Methods. Each value is expressed as mean ± S.E. Statistical difference was evaluated by Student's t test.

		Digitalis-like Activity	
	Systolic Blood Pressure (mm Hg)	Digoxin-like Immunoreactivity (pmole digoxin eq/ml)	Ouabain-like Binding Activity (pmole ouabain eq/ml)
Control (n = 10)	106±6.2	n.d.	1.23±0.16
Salt-treated (n = 10)	112.1±5.1	n.d.	1.78±0.12*
DOCA-salt-treated (n = 18)	130.6±7.8**+	0.58±0.12	4.96±0.88**+

*: different from control, $p<0.05$
**: different from control, $p<0.01$
+: different from salt-treated, $p<0.05$
n.d.: not detected

Digitalis-like activity in plasma was determined by employing radioimmunoassay for digoxin. In both control and salt-treated rats, digoxin-like immunoreactivity was not detected in plasma (less than 0.1 pmole digoxin equivalent/ml). In DOCA-salt treated rats digoxin-like immunoreactivity detected in all rats and the mean value ± S.E. was 0.58±0.12 pmole digoxin equivalent/ml. In some rats digoxin-like immunoreactivity was measured after a week treatment of DOCA and was not detected.

Activity in plasma to replace digitalis binding to its receptor, presumably the Na^+-K^+ ATPase, was assessed by measuring inhibition of ^3H-ouabain binding to brain synaptosomal fraction. Ouabain-like binding activity was detected in all rats tested. In salt-treated rats, mean value ± S.E. of ouabain-like binding activity was 1.78±0.12 pmole ouabain equivalent/ml, which was slightly but significantly higher ($p<0.05$) than that of control rats (1.23±0.16 pmole ouabain equivalent/ml). In DOCA-salt treated rats, ouabain-like binding activity was four times higher (4.96±0.88 pmole ouabain equivalent/ml) than that of control rats. These results indicated that circulating digitalis-like activity was increased in DOCA-salt treated rats.

Further characterization of this digitalis-like substance in plasma was studied using pooled plasma from DOCA-salt treated rats. Samples corresponding to ten milliliter of pooled plasma were applied to a column of Sephadex G-25. The results of fractionation is shown in Figure 1. When digoxin-like immunoreactivity in each fraction was measured, two peaks were observed. The first peak was observed in fractions 26 and 27 and the second peak, which was detected in fractions 31 to 34, contained much higher digoxin-like immunoreactivity. Approximately 70% of total digoxin-like immunoreactivity was eluted in peak 2. The effect of various amount of eluate in peak 2 on binding of radioactive digoxin to its antibody was similar to that of digoxin (data not shown). Ouabain-like binding activity in each fraction was also assayed after desalting procedure. As shown in Figure 1, there was

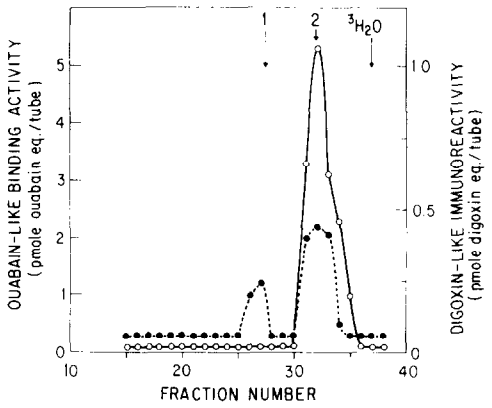

Figure 1. Results of chromatography with a Sephadex G-25 column. Digoxin-like immunoreactivity (●---●) and ouabain-like binding activity (o——o) were measured in each fraction.

only one large peak, which was eluted in fractions 31 to 35. No activity was detected in fractions 26 and 27.

The Na^+-K^+ ATPase inhibitory activity in fractions corresponding peaks 1 and 2 was also determined. Fractions from either peak 1 or 2 were pooled respectively and desalted. Na^+-K^+ ATPase inhibitory activity was assayed using brain synaptosomal fraction (7). As shown in Table 2, Na^+-K^+ ATPase activity was inhibited by only fractions from peak 2. Thus, digoxin-like immunoreactivity in peak 2 co-eluted with digitalis-like bioactivity. In contrast, the digoxin-like substance eluted in peak 1 seemed to be biologi-

Table 2. Effect of Ouabain and Digoxin-like Substance on Na^+-K^+ ATPase Activity

	Na^+-K^+ ATPase Activity (% of control)
Control	100
Ouabain	
10^{-7} M	95
10^{-6} M	88
10^{-5} M	60
Digoxin-like Substance	
peak 1	104
peak 2	86

Na^+-K^+ ATPase activity was measured in the presence of either various amounts of ouabain or digoxin-like substance equivalent to 0.1 pmole digoxin and each value was expressed as percent of control. Values were mean of two determinations and representative of three different experiments.

cally inactive (Table 2). To determine whether the digoxin-like immunoreactivity eluted in peak 1 was a complex of active substance with a peptide, digoxin-like immunoreactivity in peak 1 equivalent to 0.5 pmole digoxin was applied to the same column after treatment with protease. There was no change in elution pattern of peak 1 by protease treatment (data not shown). On the other hand, digitalis-like bioactivity as well as immunoreactivity eluted in peak 2 was resistant to protease treatment (data not shown). It was not changed by treatment with 1 M acetic acid. However, both digitalis receptor binding activity and Na^+-K^+ ATPase activity disappeared after treatment with 0.1 M sodium hydroxide for 10 min, even though digoxin-like immunoreactivity was not lost (data not shown).

Discussion

In the present study, digoxin-like immunoreactivity was detected in plasma from rats with DOCA-salt hypertension. Digitalis-like binding activity was also increased in the plasma from this group. The reason for the absence of digoxin-like immunoreactivity in control and salt-treated rats may be limited sensitivity of the radioimmunoassay. From the findings that column fractions containing digitalis receptor binding activity (Fig. 1) had both digoxin-like immunoreactivity and Na^+-K^+ ATPase inhibitory activity (Table 2), it is clear that an endogenous digitalis-like substance is increased in plasma of DOCA-salt hypertension rats. These observations support previous reports (8-10) which indicate that the Na^+-K^+ ATPase of vascular smooth muscle is partially inhibited by a circulating Na^+ pump inhibitor in DOCA-salt hypertension. In addition, the amount of a digitalis-like substance was also increased in the plasma of salt-treated rats (Table 1). These data suggest that secretion of this substance may be stimulated by sodium loading alone. These results are also in agreement with the reports by Gruber and co-workers showing that sodium loading results in an increase in digoxin-like substance in plasma (6) and that this digoxin-like substance, which sensitizes vascular smooth muscle (11), is increased in plasma of hypertensive monkeys (12).

The nature of the digitalis-like substance has not yet been elucidated and there have been arguments whether it is peptide or not (3,6,14). In the present study, digitalis-like substance was resistant to proteolytic enzymes and acid hydrolysis, however, the activity was lost by alkaline treatment, which indicate that it is unlikely to be a peptide. Although many studies have demonstrated that natriuretic hormone has digoxin-like immunoreactivity (6,14,15), whether it is structurally related to digoxin is still an open question. The anti-digoxin antibody employed in this study is raised against digoxigenin, a steroid portion of digoxin, and has little cross-reactivity with other cardiac glycosides. The antibody also blocks the action of digoxin (5). The fact that digoxin-like substance in plasma reacts with anti-digoxin antibody in the similar manner as digoxin suggests the possibility that this substance resembles digoxin not only in mode of action but also in its structure. Further study is necessary to determine the structure of this substance.

The result shown in Figure 1 also indicates that approximately 30% of total digoxin-like immunoreactivity, which is eluted in peak 1, is not associated with biological activity (Table 2). At the present time the relation between these two peaks of immunoreactivity is not known. The existence of a substance in plasma which cross-reacts with digoxin antibodies but does not inhibit the Na^+-K^+ ATPase indicates that in studies, in which digitalis-like activity is assessed solely by using radioimmunoassay for digoxin, the data should be interpreted with considerable caution.

Acknowledgements

The author is grateful and indebted to Dr. Etsuro Ogata (University of Tokyo School of Medicine, Tokyo, Japan) for his support and encouragement, and Dr. Howard Rasmussen (Yale University School of Medicine, New Haven, Connecticut) for his advice in preparing this manuscript.

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