Increased Circulating Levels of an Endogenous Digoxin-Like Factor in Hypertensive Monkeys

KENNETH A. GRUBER, PH.D., LAWRENCE L. RUDEL, PH.D., AND BILLY C. BULLOCK, D.V.M.

SUMMARY An endogenous, immunoreactive digoxin-like factor (endoxin) was measured in the plasma of nonhuman primates with hypertension. Both normotensive and hypertensive rhesus monkeys had levels of endoxin that significantly correlated with their systolic or diastolic blood pressure. Vervet monkeys with experimentally produced chronic Goldblatt hypertension had significantly elevated endoxin, but not plasma renin. These data suggest that increased plasma endoxin may be a contributing factor in the development of hypertension. (Hypertension 4: 348-354, 1982)

KEY WORDS • digitalis-like factor • sodium-potassium ATPase inhibition • natriuretic hormone

Several investigators have proposed that a circulating inhibitor of cardiovascular sodium-potassium adenosine triphosphatase ([Na⁺ + K⁺] ATPase) may be a causative factor in some forms of hypertension.¹ ² The initial evidence for a (Na⁺ + K⁺) ATPase defect came from data indicating a blunted vasodilatory effect of potassium infusions in both experimental⁵ ⁶ and clinical essential hypertension.⁷ This was followed by direct measurement of the cardiovascular enzyme in models of renovascular hypertension.¹ These experiments revealed an inhibition of cardiovascular (Na⁺ + K⁺) ATPase, an effect that could be duplicated in vitro by application of cardiac glycosides. These data suggested the presence of a circulating inhibitor of the enzyme.

Both theoretical⁸ and experimental⁹-¹⁰ evidence supports the concept that inhibition of (Na⁺ + K⁺) ATPase can increase the contractile force of vascular smooth muscle. The mechanism of action could involve the compensatory inhibition or reversal of a sodium-calcium exchange pump due to increased levels of intracellular sodium.² This would result in elevated intracellular calcium (Ca), potentiating contraction. The term "digitalis-like factor" has been used to describe this proposed substance since its physiological action, inhibition of (Na⁺ + K⁺) ATPase and potentiation of vascular smooth muscle contraction, would be similar to that of the cardiac glycoside class of drugs.¹¹ Interestingly, many different clinical assays for cardiac glycosides have detected an endogenous interfering substance in the plasma from patients with cardiovascular or renal disease.¹²-¹⁴ In addition, the presence of a receptor for cardiac glycosides suggests the existence of an endogenous ligand.

These data led us to investigate the presence of a physiological regulator of cardiovascular and renal (Na⁺ + K⁺) ATPase,¹⁵ a factor that we felt was the interfering substance in cardiac glycoside clinical assays. We have recently published¹⁶ a preliminary report on the purification of a compound in plasma, apparently peptidic in nature, which is an inhibitor of (Na⁺ + K⁺) ATPase and which shares immunological determinants with the cardiac glycoside digoxin (a specific compound of the digitalis class of drugs). This latter feature allows its assay with antidigoxin.
DIGITALIS-LIKE FACTOR IN HYPERTENSION/Gruber et al. 349

antibodies. The historic precedent for using antidiogoxin antibodies to detect an endogenous substance with cardiac glycoside-like biological activity is found in the work of Spector and his collaborators.17-19 These investigators proposed and demonstrated that some antibodies to drugs can serve as surrogate receptors for endogenous substances that have similar biological activity to the drug, presumably due to similarities in determinants for antibody and receptor binding. The ability of antibody binding sites to closely resemble a receptor has been independently confirmed by Hough and Edwardson,20 working with proteinous and nonproteinous sweet substances.

We have demonstrated that the levels of this endogenous digoxin (endoxin) increase after extracellular volume expansion with normal saline, suggesting it may be the putative natriuretic hormone. With the availability of an endoxin radioimmunoaassay (RIA) and the evidence for a digitalis-like factor in hypertension, it seemed logical to ask the question: "Are endoxin levels elevated in hypertension?" We now report the results of our study of endoxin in rhesus monkeys with spontaneously elevated blood pressure and in African green vervet monkeys with chronic two kidney-one clip (2K1C) Goldblatt hypertension.

Materials and Methods

Male rhesus (Macaca mulatta) and African green vervet (Cercopithecus aethiops) monkeys were obtained from the Arteriosclerosis Research Center of the Bowman Gray School of Medicine. The rhesus monkeys (n = 10) were part of a study on the effects of high (plasma levels of ~ 300 mg/dl) and low (plasma levels of ~ 200 mg/dl) cholesterol diets on arteriosclerosis. The precise details of this dietary regime have been previously published.21 The "hypertensive" monkeys were randomly distributed in both groups (two of five hypertensives were in the high cholesterol group). Spontaneous hypertension has previously been described in this species in their natural environment (see below). Rhesus monkeys were housed in groups of five and had their blood pressures taken at monthly intervals by a cuff and a 1010 Arteriosonde (Roche Medical Electronics). Each animal was first injected with 15 mg/kg of ketamine, and its blood pressure was taken at 10, 12, and 15 minutes and averaged. Ketamine has been previously shown to be a drug that will not significantly change relative blood pressure readings in monkeys.22,23 These indirect blood pressures averaged 15 to 20 mm Hg below direct measurements. At the time of sacrifice, the monkeys were approximately 8 to 10 years old.

Male African green vervets (n = 14), approximately 4 years of age, had a 0.7 mm Goldblatt clamp placed on one renal artery in late 1977 or early 1978 (contralateral kidney intact). Control vervets (n = 16) were sham-operated at about the same time. Prior to the initiation of the experiment, there was no significant difference in the blood pressures of those vervets chosen for the Goldblatt operation compared to the controls. The vervets were housed in individual cages and fed semipurified diets, with half the animals of each group receiving a high cholesterol diet.24 There was no statistically significant correlation between the level of dietary cholesterol and blood pressure. Blood pressures were taken monthly by the ketamine anesthesia protocol mentioned above, using a Model 1245 Dinamap Research Monitor (Applied Medical Research Corporation, Tampa, Florida). These pressures also averaged 15-20 mm Hg below direct measurements.

Venous blood for endoxin assays was obtained from the rhesus monkeys prior to sacrifice, and from the vervets (in late 1980 or early 1981) before or after a blood pressure measurement session. All animals were fasted overnight before blood samples were taken. Blood was drawn into iced heparinized 10 ml vacutainer tubes. The plasma was isolated in a refrigerated centrifuge, and processed for endoxin RIA.

In brief, the technique involved diluting the plasma 1:1 with distilled water, adjusting the pH to 5.5 with acetic acid, and immersing the sample in boiling water for 10 minutes to precipitate large proteins. This protocol has been used to isolate a circulating inhibitor of toad bladder sodium transport from volume-expanded dog plasma.25 After the proteins were removed by centrifugation (12,000 X g for 15 minutes), the supernatant was Diafiltered through Amicon UM-10 molecular sieve membranes (10,000 dalton exclusion limit) with 0.05M acetic acid. The diacritate was lyophilized, and stored at -60°C for eventual RIA. For the RIA, the diacritate was dissolved in 500 µl of distilled water and the pH adjusted to 7.6 with 1N sodium hydroxide. In the RIA, digoxin standards, blank tubes (in pH 7.6 phosphate buffered saline), and diafiltrates were incubated with 125I-digoxin (Burroughs Wellcome, Greenville, North Carolina) and a goat antidiogoxin antibody for 2 hours at 37°C. Rabbit antigoat T-globulin was then added to the reaction mixture and incubated for an additional hour. Samples were then centrifuged, decanted, and the pelleted radioactivity determined in a T-counter. The precise details of this assay have been published.26

Venous blood for renin and renin substrate assays were drawn into iced 10 ml vacutubes containing EDTA. Samples were obtained from fasted African green vervets in early 1981. The collected blood was immediately placed in an ice slurry, the plasma isolated in a refrigerated centrifuge, and quickly frozen on dry ice for storage. Renin activity was determined by the method of Gould et al.27 and the results reported in World Health Organization international renin units (IRU). Renin assays were obtained in only six of seven hypertensive Goldblatt vervets due to the death of one monkey from malignant hypertension. In both the endoxin and renin determinations, the sample collection and assays were performed blindly.

In our studies hypertension was defined as a mean blood pressure (MBP) two standard deviations (2 SD) above the average of a control group. The sham-operated vervets were considered to be a normotenive control to which the Goldblatt group was com-
pared. In the case of the rhesus monkeys, their blood pressures divided them into two groups with a separation of 2 SD. In vervets, blood pressure evaluation was by the average of the MBP from the last 10 monthly readings. In rhesus monkeys, blood pressure evaluation was by the average of the last five monthly readings. It was possible to average fewer readings in the case of rhesus monkeys since they showed more consistent blood pressure measurements, perhaps due in part to their larger size.

Statistical evaluation was by Student's t test (unpaired, unequal variance), correlation coefficients (two-tailed), and Duncan's new multiple range test. Significance was considered to be p < 0.05.

Results

Rhesus Monkeys

The rhesus monkeys could be divided into high and low blood pressure groups. Table 1 shows the blood pressures of these monkeys, with "hypertensives" averaging a mean indirect pressure of about 100 mm Hg (15-20 mm Hg below direct pressures), and normotensives averaging 80 mm Hg. Table 2 demonstrates that the division of rhesus monkeys into blood pressure groups also divides them by their plasma endoxin levels. In both the normotensive and hypertensive rhesus plasma samples, it was possible to significantly correlate endoxin to systolic, diastolic, and mean blood pressure. Figure 1 shows the plots of plasma endoxin for these 10 monkeys vs their MBP averaged for the last 5 months of their life. Correlation coefficients were also calculated using the last recorded blood pressure (the time at which the plasma endoxin sample was drawn) and an average of the last 10 months. While all the correlations are highly significant (r > 0.8), the best one is obtained with an average of the last 5 months of the animal's life (r = 0.94).

African Green Vervet Monkeys

Taken as one group, vervets with Goldblatt clamps had significantly elevated blood pressures (table 3) and plasma endoxin (table 4) when compared to sham-operated controls (both comparisons by Student's t test). However, it is well known that not all animals with Goldblatt clamps develop hypertension. Analysis of the blood pressure data revealed that the Goldblatt vervets fell into two populations: half (n = 7) had a MBP (107 mm Hg) at least 2 SD above that of the sham-operated controls (75 mm Hg), and half (n = 7) had a MBP (69 mm Hg) not statistically different from that of the controls (table 3). Thus, 50% of the animals with a Goldblatt kidney developed hypertension. This is similar to what has been reported in other species.

Since our blood pressure and surgical classification resulted in three groups of monkeys (controls, normotensive Goldblatt, and hypertensive Goldblatt), the statistical analysis of plasma endoxin was by Duncan's new multiple range test as well as by t test. This demonstrated that the plasma endoxin level of both Goldblatt groups was significantly elevated over that of the controls (table 4), although the mean of the hypertensives was somewhat higher than that of the normotensive Goldblatt group. Interestingly, two Goldblatt vervets classified as nonhypertensive were in fact only 5-7 mm Hg (1 SD above controls) away from the blood pressure cutoff for hypertension. In these

TABLE 1. Rhesus Blood Pressures

<table>
<thead>
<tr>
<th>Rhesus group</th>
<th>Mean blood pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normotensive (n = 5)</td>
<td>82.0 ± 2.5</td>
</tr>
<tr>
<td>Hypertensive (n = 5)</td>
<td>105.8 ± 4.4*</td>
</tr>
</tbody>
</table>

The mean blood pressure value is the average of the last five monthly readings ± standard error (SE).

*Statistically different from respective controls (p < 0.01 by Student's t test); the mean blood pressure of each member of the hypertensive group falls at least 2 standard deviations above the average of the respective normotensive group.

TABLE 2. Rhesus Plasma Endoxin

<table>
<thead>
<tr>
<th>Rhesus group</th>
<th>Plasma endoxin (pg digoxin equivalents per ml of plasma ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normotensive (n = 5)</td>
<td>26.2 ± 4.5</td>
</tr>
<tr>
<td>Hypertensive (n = 5)</td>
<td>80.0 ± 11.3*</td>
</tr>
</tbody>
</table>

*Significantly different from normotensive group (p < 0.01 by Student's t test).

FIGURE 1. Plots of mean blood pressure averaged for 5 months vs endoxin levels in rhesus monkeys.
TABLE 3. Blood Pressure in Vervets with a Unilateral Goldblatt Clamp

<table>
<thead>
<tr>
<th>Vervet group</th>
<th>Mean blood pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated control (n = 16)</td>
<td>75.5 ± 3.5</td>
</tr>
<tr>
<td>All Goldblatt-clamped (n = 14)</td>
<td>88.1 ± 6.1*</td>
</tr>
<tr>
<td>Normotensive Goldblatt-clamped (n = 7)</td>
<td>68.6 ± 4.1</td>
</tr>
<tr>
<td>Hypertensive Goldblatt-clamped (n = 7)</td>
<td>107.7 ± 4.2†</td>
</tr>
</tbody>
</table>

The mean blood pressure value for each group is the average of 10 monthly readings ± SE.
*Significantly different from controls (p < 0.05).
†Significantly different from controls (p < 0.01).
†Two standard deviations above controls.

monkeys plasma endoxin levels approached the mean of the hypertensive Goldblatt vervets. There was no correlation between blood pressure and endoxin in the normotensive or hypertensive vervet monkeys. When both groups were combined, a statistically significant correlation was found (p < 0.02). However, the r value was only 0.52, which suggests that the significance was due to the large difference in blood pressures and endoxin between the normotensive and hypertensive groups, in effect drawing a straight line between two points. This is in contrast to the highly significant correlation seen within each group of rhesus monkeys.

Plasma renin assays showed no statistical difference between controls and the hypertensive or normotensive Goldblatt vervets. However, three of six hypertensive Goldblatts had extremely elevated renin, while the remaining ones were similar to controls. Figure 2 demonstrates the variability in plasma renin activity in the hypertensive Goldblatt monkeys vs the other groups. There was no correlation between renin levels and blood pressure in any of the three groups of vervets.

**TABLE 4. Plasma Endoxin in Vervets with a Unilateral Goldblatt Clamp**

<table>
<thead>
<tr>
<th>Vervet group</th>
<th>Plasma endoxin (pg digoxin equivalents per ml ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated control (n = 16)</td>
<td>73.7 ± 13.0</td>
</tr>
<tr>
<td>All Goldblatt-clamped (n = 14)</td>
<td>165.7 ± 25.6*</td>
</tr>
<tr>
<td>Normotensive Goldblatt-clamped (n = 7)</td>
<td>138.3 ± 34.9†</td>
</tr>
<tr>
<td>Hypertensive Goldblatt-clamped (n = 7)</td>
<td>197.7 ± 36.3†</td>
</tr>
</tbody>
</table>

*Significantly different from controls (p < 0.01 by Student's t test).
†Significantly different from controls (p < 0.05 by Student's t test).
†Significantly different from controls (p < 0.01 by Duncan's multiple range test).

**Discussion**

Modi and Chakravarti28 have demonstrated that spontaneous hypertension in rhesus monkeys is not associated with increased levels of angiotensin II, or renal pathology. Histological examination of kidneys from normotensive and hypertensive rhesus monkeys in our study has also revealed no consistent pattern of renal pathology (unpublished data). Thus, spontaneous increases of blood pressure in this species of nonhuman primates have certain features in common with human essential hypertension (i.e., presumably low renin with no renovascular pathology). Overbeck et al.7 have presented data indicating depressed (Na+ + K+) ATPase activity in vascular smooth muscle of humans with essential hypertension. Therefore, our data indicating elevated endoxin in spontaneously hypertensive rhesus monkeys not only suggest a role for this putative hormone in the pathophysiology of the disease, but also a further parallel between the idiopathic forms of elevated blood pressure in human and nonhuman primates.

The 2K1C form of Goldblatt hypertension is usually thought of as a high renin model of hypertension.29 However, there is basic27, 30-34 and clinical35, 36 evidence that constriction of one renal artery does not always lead to elevated plasma renin activity, or to a form of hypertension susceptible to agents that block
the renin-angiotensin system. Swales and co-workers have suggested a dual mechanism for the development of renovascular hypertension, since the chronic phase of 2K1C Goldblatt hypertension is not necessarily associated with elevated renin and angiotensin. They hypothesize that the development of renal pathology may lead to sodium retention, which reduces renin activity; this in turn may lead to a form of hypertension more akin to the one-kidney one clip model, i.e., a "volume-expanded" form of hypertension in which endoxin is elevated. Support for this hypothesis has been provided by studies of plasma angiotensin II in acute and chronic renovascular hypertension in rhesus monkeys. It should be noted, however, that endoxin levels were equally elevated in all hypertensive Goldblatt vervets, although only half had normal renins. Thus, the precise regulatory mechanism for elevated endoxin in this form of Goldblatt hypertension is not well defined.

Elevation of endoxin in Goldblatt clamped vervets whose blood pressure is normotensive by our criteria was at first somewhat perplexing. To our knowledge, ours is the first report of 2K1C Goldblatt animals who do not become hypertensive. In a preliminary communication, we showed by saralasin infusion that 2K1C vervets shift a significant amount of their blood pressure regulation to angiotensin II, although their renin levels are not elevated. This suggests that their vasculature may be sensitized to angiotensin II. In addition, they have significantly depressed baroreceptor reflex gain. Both of these characteristics are also found in the hypertensive vervets. Thus, our initial findings suggest that normotensive Goldblatt vervets are not "normal," but in fact share alterations in cardiovascular regulation that might be thought of as predisposing to the development of hypertension. An intriguing question is: Why don't these animals develop hypertension? We are currently conducting studies to address this problem.

Recently, Schreiber et al. have independently confirmed some of our findings by demonstrating that serum levels of digoxin-like immunoreactivity increase in rats with coarctation of the abdominal aorta proximal to the renal arteries. This model of hypertension is probably not associated with elevated angiotensin II, and there is evidence for undefined humoral factors in the pathogenesis of this disease. Schreiber et al. were able to relate digoxin-like immunoreactivity to left ventricular or total cardiac weight in the hypertrophied hearts of their rats. It can thus be observed in three different hypertensive models that endogenous digoxin immunoreactivity is elevated.

As previously noted, many investigators have proposed the presence of a circulating inhibitor of (Na+ + K+) ATPase to account for the inhibition of this enzyme in cardiovascular tissue in various forms of hypertension. Haddy and Overbeck have proposed that the putative natriuretic hormone (NH) might be this inhibitor since: 1) volume expansion will increase NH levels, and they have detected the presence of an inhibitor in volume-expanded forms of hypertension; 2) Gonick et al. have shown that volume expansion will increase circulating levels of an (Na+ + K+) ATPase inhibitor; and 3) Mizukoshi and Michelakis have demonstrated that salt loading will increase bioassayable levels of a vascular smooth muscle sensitizing factor.

In a previous communication, we first presented evidence that endoxin may be the putative NH. Our reasons for proposing this include 1) endoxin has the identical high performance liquid chromatography (HPLC) retention time as a factor with a NH characteristic (inhibition of toad bladder sodium transport); 2) in our laboratory we have correlated our toad bladder bioassay results with the presence of two fluorescently positive peaks on reverse-phase HPLC, and have shown that the immunological and (Na+ + K+) ATPase inhibitory characteristics of endoxin are also found in two similar HPLC peaks; and 3) the level of the substance detected by our bioassay, RIA, and enzyme assay increases after salt loading. Bohan et al. have provided support for our hypothesis by demonstrating in volume-expanded dogs that chromatographic fractions of urine containing bioassayable NH will also displace [3H]-ouabain from isolated renal (Na+ + K+) ATPase receptors in a competitive binding assay. The displacement curve for the NH-containing fractions paralleled that of cold ouabain.

Recently Plunkett et al. in our laboratories have shown that endoxin-containing HPLC extracts will potentiate the contraction of third order (20–40 μ) arterioles in response to a standard dose of norepinephrine. This segment of the vasculature is thought to be important in blood pressure regulation. Further confirmation of the presence of a vascular sensitizing factor was provided by Szakacs and Juhasz, and Self et al. The former workers demonstrated a blood pressure sensitizing factor in persons genetically predisposed to developing hypertension, before any blood pressure rise could be detected, suggesting it could be a risk factor for the development of the disease. It should be noted that there is considerable evidence for altered vascular reactivity in borderline experimental and essential hypertension. These findings are not associated with activation of the renin-angiotensin system, but are compatible with increased circulating levels of a vascular sensitivity potentiating factor.
A final piece of evidence for the involvement of endoxin in blood pressure elevation is from rats with electrolytic lesions of the anteroventral third ventricle area (AV3V) of the hypothalamus. AV3V lesioned rats will not develop many forms of renovascular hypertension. In a preliminary study, we demonstrated that lesioned rats cannot excrete an isotonie saline load and do not have detectable toad-bladder sodium transport inhibitory activity in their plasma. Independent support that this lesion may prevent the release of a digitalis-like substance has been provided by Pannani et al. They showed that volume-expanded AV3V lesioned rats did not have an inhibition of vascular (Na\(^+\) + K\(^+\)) ATPase, whereas volume-expanded sham-lesioned rats did.

The final proof of the hypothesis drawn from these data awaits the structural elucidation of endoxin and experiments performed with appropriate analogs.

**Acknowledgments**

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