Evidence for Increased Levels of a Circulating Ouabainlike Factor in Essential Hypertension

GIUSEPPE A. SAGNELLA, JULIA C. JONES, ANGELA C. SHORE, NIRMALA D. MARKANDU, AND GRAHAM A. MACGREGOR

SUMMARY The effect of plasma from normotensive and hypertensive subjects on the binding of \(^{[3]H}\)ouabain on human erythrocytes was investigated. The binding of \(^{[3]H}\)ouabain on human erythrocytes was saturable and highly specific; linear Scatchard plots indicated the presence of a single type of binding site. Human plasma decreased the binding of \(^{[3]H}\)ouabain on its receptor to a greater extent than could be accounted for by the plasma potassium concentration. The level of this circulating ouabainlike factor (or factors) was quantitated using a radioreceptor assay. Plasma from 22 hypertensive subjects (systolic blood pressure > 160 mm Hg or diastolic blood pressure >90 mm Hg) displayed higher levels than that from 24 normotensive subjects; furthermore there was a positive and significant correlation (r = 0.42, n = 46, p < 0.004) between the ouabainlike content and the individual subject's systolic blood pressure. The receptor assay described is relatively simple and should be useful for further work on the nature and clinical importance of the endogenous ouabainlike factor. (Hypertension 8: 433-437, 1986)

KEY WORDS • hypertension • Na\(^+\)-K\(^+\) pump inhibitor • ouabainlike factor • radioreceptor assay

INCREASING evidence indicates that a high salt intake may predispose to the development of high blood pressure\(^1\) and that the high blood pressure may be secondary to increased levels of a circulating Na\(^+\) transport inhibitor.\(^2,3\) However, the exact chemical nature of this transport inhibitor remains unresolved. Indeed, the existence of this factor (or factors) recently has been questioned.\(^4,5\) Nevertheless, previous work in humans and in experimental models of hypertension in animals (for recent review, see References 2, 3, 7), in particular the observation that plasma from hypertensive subjects inhibited ouabain-sensitive Na\(^+\) transport in white blood cells,\(^6\) suggests that the inhibitor may have ouabainlike properties. This possibility is supported by recent work on the effects of plasma extracts from hypertensive and normotensive subjects on the binding of cardiac glycosides on human erythrocytes.\(^7,8\) Since a Na\(^+\)-K\(^+\) pump inhibitor potentially could interact and displace cardiac glycosides from the glycoside receptor, the present study investigated the effects of plasma from normotensive and hypertensive subjects on the binding of radioactive ouabain on human erythrocytes.

Subjects and Methods

The investigations were carried out in normotensive subjects and subjects with essential hypertension without clinical or laboratory evidence of cardiac failure or renal insufficiency who had been referred to the blood pressure unit for further investigation by their local practitioner. None of the normotensive subjects were receiving medication, and none of the hypertensive subjects had received drug treatment for at least 2 weeks before the study. Subjects' supine blood pressures were measured in the same arm, using semiautomatic ultrasound sphygmomanometers (Arteriosonde; Roche, Nutley, NJ, USA) with attached recorder, and thus were free from observer bias. Blood pressures were calculated as the mean value of five recordings taken every 1 to 2 minutes. The normotensive subjects consisted of 24 healthy hospital volunteers (21 whites, 13 men, 8 women; 3 blacks, 2 men, 1 woman) with a supine systolic blood pressure less than 160 mm Hg and a diastolic blood pressure less than 90 mm Hg. The mean age of the group was 43 years (range, 19–70 years). The systolic and diastolic blood pressures of the hypertensive subjects were consistently greater.
than 160 or 90 mm Hg, respectively, and the group consisted of 22 subjects, including 15 whites (9 men, 6 women) and seven blacks (3 men, 4 women). The mean age of the hypertensive subjects was 49 years (range, 26–67 years). Dietary sodium intake was unrestricted in all subjects. All subjects gave informed consent, and studies were approved by the local ethical committee.

Blood samples were collected in the morning (between 1000 and 1200) by venous puncture after the subjects had been sitting for 5 to 10 minutes. Blood samples for the measurement of ouabainlike activity were collected in heparin tubes; after centrifugation to remove blood cells, the plasma was transferred into plain plastic tubes and stored frozen at \(-20^\circ\text{C}\).

Biochemical estimations were performed on coded samples in batchwise assays. Each assay contained a roughly equal number of normotensive and hypertensive plasma samples. The potassium and sodium contents of the plasma samples were measured by flame photometry. Plasma renin activity was measured by radioimmunoassay as described previously.10

To calculate [\(^{3}\text{H}\)]ouabain saturation binding on human red blood cells, blood (10 ml) was collected from normotensive subjects in heparin tubes and the blood cells were washed three times in 2 volumes of tris(hydroxymethyl)aminomethane (Tris) buffer (containing NaCl, 130 mM; sucrose, 20 mM; glucose, 10 mM; Tris, 10 mM; pH 7.4 at 37°C). The Buffy coat was removed, and the packed red blood cells were resuspended in the Tris buffer to a hematocrit of approximately 15%. A stock solution of ouabain (10 mM) was made in 10% ethanol and appropriately diluted in physiological saline. The [\(^{3}\text{H}\)]ouabain (35 Ci/mmol) was obtained from Amersham International (Amer- sham, UK). Aliquots of the radioactive material (25 \(\mu\)l) were transferred into a glass tube, the solvent was evaporated under nitrogen, and the [\(^{3}\text{H}\)]ouabain was redissolved in saline and used immediately. The saturation experiments were performed by incubating 250-\(\mu\)l samples of the erythrocyte suspension with 50-\(\mu\)l aliquots of serially diluted (in saline) radioactive ouabain and 50-\(\mu\)l aliquots of saline or cold ouabain (0.1 mM) at 37°C for 4 hours. The cells then were washed three times with 800-\(\mu\)l portions of ice-cold saline, the final pellet of red blood cells was resuspended with 200 \(\mu\)l of saline, and 100-\(\mu\)l aliquots were transferred into liquid scintillation vials. These samples were then solubilized with toluene (500 \(\mu\)l), decolorized with 30% hydrogen peroxide (100 \(\mu\)l), and left at room temperature for 15 minutes. Then 4 ml of Dimilune was added, and the amount of [\(^{3}\text{H}\)]ouabain bound to the red blood cells was counted in a liquid scintillation counter after the vials had been stored in the dark overnight. All assays were performed in duplicate. Specific binding was calculated by subtracting the nonspecific binding counts (in the presence of 0.1 mM ouabain). The nonspecific binding counts were usually less than 10% of the total counts.

Dowex resin (50-W-X8, BDH Chemicals, London, England) was batch-treated with 100 volumes of 1M NaOH and then extensively washed in distilled water to neutral pH. Frozen plasma samples were thawed and centrifuged briefly to remove particulate matter, and 1-ml aliquots then were added to 600 mg (total weight) of the resin and left at 4°C for 30 minutes with occasional mixing. The plasma was separated from the resin and analyzed immediately for its effects on the binding of [\(^{3}\text{H}\)]ouabain on human erythrocytes.

To study the effects of human plasma on [\(^{3}\text{H}\)]ouabain binding on human red blood cells, blood (20 ml) was obtained in heparin tubes from normotensive subjects (O blood group). A suspension of red blood cells was prepared as just described, except the concentration of Tris buffer was 50 mM instead of 10 mM. The effects of plasma were tested by incubating 200-\(\mu\)l Dowex-treated plasma samples with the red blood cell suspension (250 \(\mu\)l) for 2 hours at 37°C. Radioactive ouabain (50 \(\mu\)l, to give a final concentration of 19 nM) then was added, and all tubes were reincubated for a further 2 hours. All samples were tested in duplicate, and appropriate dose-response curves for ouabain and potassium (seriously diluted in 200 \(\mu\)l saline) were included in every experiment. At the end of the incubation, the red blood cells were washed three times with 800 \(\mu\)l wt/vol of ice-cold saline and 10% trichloroacetic acid (200 \(\mu\)l) was added to the final pellet of red blood cells. The tubes then were vigorously mixed using a vortex, left at 4°C for 5 to 10 minutes, and centrifuged to sediment the precipitated protein. Finally, 150-\(\mu\)l samples of the clear supernatant were used to measure the amount of bound radioactive ouabain in 3.5 ml of Picofluor 30 liquid scintillation fluid.

Statistical analysis was performed using the appropriate programs of the Statistical Package for the Social Sciences12 as described in Results. Results are expressed as means ± standard deviation (SD).

Results

The amount of radioactive ouabain specifically bound on the human red blood cells increased with increasing concentration of total [\(^{3}\text{H}\)]ouabain in a typical hyperbolic manner (Figure 1A). The amount of [\(^{3}\text{H}\)]ouabain bound was decreased in the presence of unlabeled ouabain, indicating that the binding was saturable and could be displaced by the specific ligand (see Figure 1A). A Scatchard plot of the data was linear, thus indicating the presence of a single class of receptor sites (Figure 1B). The dissociation constant for the interaction between ouabain and its receptor was estimated from the saturation data by nonlinear regression.13 The results obtained are well in agreement with previous findings14-17; the mean dissociation constant value in four normotensive subjects was 5.04 ± 1.96 nmol/L.

Radioactive ouabain could be displaced in a dose-dependent manner by nonradioactive ouabain (Figure 2A). The dose-response curve was effectively linearized by a logit-log transformation.18 The slope of the line estimated by linear regression was -0.87 ± 0.11 (\(n = 3\)). The precision profile throughout the standard
OUABAINLIKE FACTOR IN ESSENTIAL HYPERTENSION/Sagnella et al. 435

FIGURE 1. A. Effect of ouabain on \(^{3}H\)ouabain binding on human red blood cells (RBC). Erythrocytes were obtained from normal subjects and incubated as described in Methods. The saturation curves are given in the absence (•) or presence of 1.43 nM cold ouabain (△). B. Scatchard plot of the \(^{3}H\)ouabain binding data shown in A.

curve showed a mean coefficient of variation of about 4% with a 95% confidence interval between 2 and 6%. The mean interassay coefficient of variation on two individual plasma samples (pretreated with Dowex resin) assayed in duplicate in three separate assays was 11.1%. Plasma potassium, however, displayed a considerable degree of inhibition of the amount of \(^{3}H\)ouabain bound on the red blood cells. To overcome interference by plasma potassium, plasma samples were treated with a potassium-selective resin (Dowex 50-W-X8), which markedly reduced the amount of potassium in the plasma (Figure 2B), and the total ouabainlike content was corrected for the amount due to the residual potassium. In most samples this amount represented a small proportion (about 10%) of the total ouabainlike content.

Plasma samples from normotensive and hypertensive subjects showed a variable degree of inhibition of the amount of \(^{3}H\)ouabain bound on human erythrocytes that could not be attributed to its potassium content. This value ranged from 4.7 to 27.3% of the total \(^{3}H\)ouabain bound. The degree of inhibition was expressed as ouabain equivalents (nmol/L) present in the original plasma samples by direct comparison with a ouabain dose-response curve. The results showed a mean for the whole group of 1.39 ± 0.72 nmol/L. The levels in the 22 hypertensive subjects (systolic blood pressure > 160 mm Hg or diastolic blood pressure > 90 mm Hg) was greater than that of the 24 normotensive (systolic < 160 mm Hg or diastolic < 90 mm Hg) subjects (1.64 ± 0.66 vs 1.16 ± 0.73 nmol/L; unpaired t test, \(p < 0.03\)).

A test of correlation was made between individual subjects’ plasma ouabainlike content and their respective blood pressures. A positive and statistically

FIGURE 2. Effect of ouabain and potassium on \(^{3}H\)ouabain binding on human erythrocytes. A. Binding of \(^{3}H\)ouabain was inhibited by increasing amounts of cold ouabain. Red blood cells were obtained from normotensive subjects. Results are means ± SD of four experiments performed in duplicate. For details, see Methods. B. Binding of \(^{3}H\)ouabain on human red blood cells was inhibited by potassium ions. The concentrations given are those in the incubation mixture set up as described in Methods. The two filled blocks indicate the potassium content in the incubation mixture when untreated plasma (0.9–2.2 mM) or Dowex-treated plasma (< 0.1 mM) was used. Results are means ± SD of four experiments performed in duplicate.
significant correlation was found with the individual subject's systolic blood pressure with a Pearson correlation coefficient of 0.42 (n = 46, p < 0.004; Figure 3). The correlation coefficient was statistically significant even when only white subjects were considered (r = 0.45, n = 36, p < 0.006). A significant correlation was also found with the mean arterial pressure in all subjects (r = 0.36, p < 0.02) and in the white subjects only (r = 0.41, p < 0.02). There was no statistically significant correlation between the ouabainlike content and the plasma renin activity in all subjects, nor was there any difference in the mean values between the low renin (plasma renin < 1.0 ng/ml/hr) and high renin hypertensive subjects. Furthermore, as determined by analysis of variance, the plasma ouabainlike content was not related to the race or sex of the subjects in the group as a whole or in the normotensive and hypertensive subjects analyzed separately.

Discussion

Essential hypertension usually is associated with an increase in total peripheral vascular resistance, which probably represents either an altered vascular design due to arterial hypertrophy or functional changes of the smooth muscle cells leading to an increase in tension. Considerable evidence has been accumulated that is consistent with the latter concept as well as with the concept that the increase in smooth muscle tension may be associated with an increase in intracellular calcium concentration in the smooth muscle cells of the arterial wall (for reviews, see References 19–21). The exact mechanism that might lead to such an increase in intracellular calcium is currently a matter of considerable debate in view of the many and interacting control mechanisms. However, in conjunction with the observed relations between salt intake and blood pressure, it has been suggested that an increase in intracellular calcium may reflect increases in the levels of a circulating Na+ transport inhibitor. The rise in the levels of this inhibitor could be related to an inherited defect in the kidney's ability to excrete Na+. The exact nature of this circulating inhibitor is unclear. Additionally, numerous studies have been unable to find evidence of a circulating Na+ transport inhibitor using a variety of methods. Rather than questioning the existence of a postulated inhibitor, these results probably reflect the use of different procedures to identify and measure the effects of plasma on the activity of the Na+-K+ pump and the limitation inherent in each particular procedure. A particularly important issue is that inhibition of isolated Na+,K+-ATPase may not necessarily reflect inhibition of the Na+-K+ pump in intact cells. On the assumption that the inhibitor has ouabainlike properties, its identification requires that it should 1) inhibit the activity of isolated Na+,K+-ATPase, 2) bind with high affinity to the glycoside binding sites, and 3) inhibit Na+-K+ pump activity in intact cells. In practice, however, and particularly when using plasma samples, the application of these criteria is not straightforward.

In addition to the possibility of nonspecific interference resulting from the ionic composition of the plasma and possibly also from plasma lipids, there are also the major problems of relative sensitivity and selectivity of the various procedures (see Reference 25 for detailed discussion). On the basis that a substance that might inhibit the Na+-K+ pump might also interact with specific cardiac glycoside binding sites, the present study investigated the effects of plasma from normotensive and hypertensive subjects on the binding of [3H]ouabain on its receptor in human red blood cells. In general, the radioreceptor procedure is a simple and quantitative method that has been used extensively to identify and measure several endogenous and pharmacological agents. However, a limitation of this method is that the receptor-ligand interaction may not distinguish an agonist from an antagonist. Nevertheless, the observation that human plasma decreased the binding of radioactive ouabain on its receptor is consistent with the presence of a ouabainlike factor that might inhibit the activity of the Na+-K+ pump. The observation that the plasma of hypertensive subjects contains a significantly higher level of this ouabainlike factor is of considerable interest, especially in view of the significant correlation with individual subjects' blood pressure (see Figure 3). However, this association does not necessarily imply that increased levels of the ouabainlike material are a cause of the hypertension, in view of the lack of evidence excluding the possibility that the high levels are, in fact, a result of the higher blood pressure. Nevertheless, the weak correlation could reflect the likely possibility that the ouabainlike factor is only one of several blood pressure-determining factors.

Although it has been suggested that circulating levels of a Na+ transport inhibitor may be relatively higher in subjects with low renin hypertension, we found no correlation between the plasma renin activity
and the levels of the ouabainlike factor in the whole group, nor were the levels higher in the low renin hypertensive group in comparison with those in hypertensive subjects with higher (angiotensin I > 1.0 ng/ml/hr) plasma renin activity.

The chemical nature of the ouabainlike material measured in the present study and its relation to a Na+ transport inhibitor measured by its effects on Na+ transport in intact white blood cells 29-31 or by other procedures used as a measure of Na+-K+ pump inhibition 32, 33 remain unclear; because of the different procedures used it is not possible to make a direct comparison. However, our results can be compared with those of Devynck and colleagues, 9-10 who also investigated the effects of plasma on the binding of [3H]ouabain on human red blood cells. Although the two procedures are similar, there are important methodological differences. Devynck et al. 9 used boiled plasma extracts, whereas untreated plasma was used in the present study. The boiling procedure might have released a protein-bound material. Interestingly, a close relation between the effects of whole plasma and of deproteinized plasma on the binding of ouabain on its receptor in human erythrocytes has been found in a more recent study. 34

In conclusion, the present results are in agreement with previous work and consistent with the view that plasma from hypertensive subjects contains greater amounts of an apparent ouabainlike factor.

References

2. de Wardener HE, MacGregor GA. The relation of a circulating sodium transport inhibitor ( "the natriuretic hormone") to hypertension. Medicine (Baltimore) 1983;62:310–326
Evidence for increased levels of a circulating ouabainlike factor in essential hypertension.
G A Sagnella, J C Jones, A C Shore, N D Markandu and G A MacGregor

Hypertension. 1986;8:433-437
doi: 10.1161/01.HYP.8.5.433

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1986 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/8/5/433

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/