Calcium Binding Capacity of Erythrocyte Membrane in Human Hypertension

Massimo Cirillo, Maurizio Trevisan, and Martino Laurenzi

on behalf of the Gubbio Study Collaborative Group

The cell membrane calcium binding capacity of genetically hypertensive rats is reduced when measured in the presence of the submicromolar calcium concentrations proper of intracellular environment. The present work, performed as an ancillary study to an epidemiological survey on an entire population, aimed to investigate the existence of a similar abnormality in human hypertension. Calcium binding to the erythrocyte membrane was measured in clinically healthy normotensive (n=12) and hypertensive individuals (n=24). For this purpose, a filtration technique was used, based on the determination of $^{40}$Ca bound to the erythrocyte membrane in the presence of free calcium concentrations (40 nmol/l and 1 μmol/l), which are similar to those of the intracellular environment. The intra-assay technical error was determined on 35 duplicate samples and, when expressed as percent of the mean, was 24.1 at the 40 nmol/l concentration and 16.8 at the 1 μmol/l concentration. Membranes of untreated hypertensive patients, at both calcium concentrations, bound significantly less calcium than the control group. Treated and untreated hypertensive individuals had comparable values of membrane calcium binding capacity. Membranes of the treated hypertensive group bound less calcium than those of the normotensive group at both calcium concentrations, but the difference was statistically significant only in the presence of 40 nmol/l free calcium. A significant positive correlation was observed between the calcium binding capacity at 40 nmol/l concentration and that at 1 μmol/l in the treated and untreated hypertensive groups (r=0.73 and 0.75, respectively; 0.51 for the normotensive group). These findings support the hypothesis that a cell membrane abnormality is detectable in some hypertensive patients. The possible pathophysiological meaning of the reduction in membrane calcium binding and its possible relation with the other disorders of cellular and systemic calcium homeostasis observed in hypertension need to be further investigated. (Hypertension 1989;14:152-155)

Calcium ions play a pivotal role in vascular smooth muscle function. Previous studies have reported altered calcium metabolism in both rat and human arterial hypertension. In genetically hypertensive rats it has been reported that, in addition to abnormalities of extracellular calcium homeostasis, the membrane calcium binding capacity in the presence of calcium concentra-

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sectional phase of the Gubbio study, and who met the following criteria: age 30–60 years, absence of any acute or chronic diseases with the exception of arterial hypertension, and normal values for routine urine and blood laboratory measurements.

Blood pressure was measured by trained medical doctors with mercury sphygmomanometers and cuffs of appropriate size. Three consecutive determinations of arterial pressure and heart rate were performed in the sitting position, the first one at least 5 minutes after application of the cuff. The average of the second and third measurements was used for the analysis. Participants were asked to refrain from eating for at least 2 hours before the visit, and from smoking and strenuous exercise for one half hour before the visit. Smoking habit was assessed by a questionnaire; information was also collected on family history of hypertension and pharmacological or dietetic treatment status. A first-void morning urine sample was obtained as a part of the electrolyte evaluation.

Persons eligible for the analysis were divided into three groups. The untreated hypertensive group included all the never-treated, clinically healthy individuals screened in the indicated period whose blood pressure was equal to or greater than 140 mm Hg systolic or 90 mm Hg diastolic. The normotensive group consisted of never-treated healthy persons with systolic and diastolic pressures equal to or lower than 139 and 89 mm Hg, respectively. They were selected on the basis of sex, age, and body mass index to match the untreated hypertensive group. The treated hypertensive group consisted of clinically healthy patients, regardless of current blood pressure level, who were receiving chronic pharmacological antihypertensive treatment.

From each participant a venous heparinized blood sample was obtained with minimal stasis, and an erythrocyte membrane homogenate was prepared according to a previously described method. The presence of rightside-out or inside-out vesicles was controlled in each preparation. The red blood cell membrane homogenate was divided into three aliquots and calcium binding to the erythrocyte membrane was measured in vitro in each aliquot by filtration technique based on determination of the $^{45}$Ca that associated to the membrane. The mean of the three values was used to characterize each individual. Each aliquot was analyzed separately in the presence of both the 40 nmol/l and the 1 µmol/l free calcium concentrations, fixed in vitro by Ca-EGTA/EGTA buffer.

The technical error of these procedures was evaluated by measurement of calcium binding in split samples of red blood cell samples from a group of 35 unselected individuals surveyed during the same period in the Gubbio Study. The laboratory was totally blinded for the clinical variables and split samples.

Statistical evaluation of the data was performed by two-tailed Student's $t$ test for unpaired observations and simple linear regression analysis.

**Results**

Under our experimental conditions and in agreement with previous observations, calcium binding to erythrocyte membranes reached the equilibrium within 1 minute at $25° C$, and it remained constant up to 60 minutes at both calcium concentrations used in the study. The binding to erythrocyte ghosts was rapidly (3 minutes) reversible by lowering the free calcium concentration with the addition of an excess of EGTA, but it was not affected by the calcium ionophore A 23187 (1 µmol/l).

Rightside-out or inside-out vesicles were never detected in the membrane homogenate prepared for calcium binding studies from red blood cell samples of participants in the Gubbio Study.

The evaluation of the intra-assay variability, performed on split red blood cell samples of 35 persons, yielded an absolute technical error of 0.26 nmol calcium/mg membrane protein at the 40 nmol concentration (24.1% of the mean calcium binding capacity observed in the paired samples); at the 1

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**Table 1. Characteristics of the Healthy Normotensive Group and Untreated and Treated Hypertensive Groups Participating in the Study**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normotensives</th>
<th>Untreated hypertensives</th>
<th>Treated hypertensives</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>48±5</td>
<td>48±5</td>
<td>52±2</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>6/6</td>
<td>6/6</td>
<td>4/8</td>
</tr>
<tr>
<td>Body mass index (kg/m$^2$)</td>
<td>26.2±1.2</td>
<td>28.2±2.1</td>
<td>27.4±1.5</td>
</tr>
<tr>
<td>Systolic pressure (mm Hg)</td>
<td>128±2</td>
<td>151±6</td>
<td>155±3</td>
</tr>
<tr>
<td>Diastolic pressure (mm Hg)</td>
<td>79±2</td>
<td>97±3</td>
<td>94±2</td>
</tr>
<tr>
<td>Pulse rate (beats/min)</td>
<td>72±3</td>
<td>74±2</td>
<td>76±3</td>
</tr>
<tr>
<td>Smokers/nonsmokers</td>
<td>6/6</td>
<td>4/8</td>
<td>7/5</td>
</tr>
<tr>
<td>Family history of hypertension (pos/neg)</td>
<td>6/6</td>
<td>7/5</td>
<td>9/3</td>
</tr>
<tr>
<td>Urinary sodium/creatinine (mmol/l/mmol/l)</td>
<td>1.22±0.28</td>
<td>1.36±0.14</td>
<td>1.03±0.21</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
Reduced calcium binding to the cell membrane has been described in two models of rat genetic arterial hypertension, 4-8 although it is not detectable in secondary forms of hypertension. 9 Postnov et al. 9 reported a lower membrane calcium binding in red blood cells of hypertensive patients, suggesting that this finding reflects an abnormality of the inner side of the membrane, although the free calcium concentrations used in that in vitro study were considerably higher than that physiologically present in normal erythrocytes. 17

In this study, calcium binding to erythrocyte membrane was measured in the presence of two calcium concentrations: the first concentration (40 nmol/l) is comparable with that of the normal intraerythrocytic environment 17 while the second (1 μmol/l) reproduces the conditions of intracellular fluid in stimulated excitable cells. 1 Under both these experimental conditions, membranes of the never-treated hypertensive group bound significantly less calcium than the control group. Hypertensive patients and controls came from the same population and had comparable characteristics. Data on the never-treated and treated hypertensive groups were analyzed separately. The difference versus the normotensive group was less significant for the treated than for the untreated hypertensive groups, but both groups with high blood pressure had comparable low values of membrane calcium binding. Overall, the results are in accordance with the hypothesis that membrane calcium binding capacity is reduced in hypertension. 9 The correlation found in the hypertensive groups between calcium binding values observed at two different concentrations indicates that individuals with low membrane bound calcium at 40 nmol/l also have reduced calcium binding capacity at the 1 μmol/l concentration. This suggests that, as previously reported in hypertensive rats, 4-8 the reduction of membrane calcium binding capacity in hypertension is linked to a low number of binding sites rather than to an altered affinity of the membrane for calcium ions.

Membrane calcium binding could be involved in the regulation of membrane permeability 18 and of transmembrane calcium fluxes. 19-24 If present also at the vascular level, as observed in spontaneously hypertensive rats, 6 the reduced membrane calcium binding capacity could lead to an increase of the smooth muscle cytoplasmatic calcium concentration through some of these mechanisms, and therefore, it could play some role in the maintenance of the high peripheral vascular resistance typical of hypertension. 1

Besides altered membrane calcium binding, several abnormalities of calcium metabolism have been reported in human and rat hypertension. Hypertension is associated with reduced renal calcium reabsorption, 2,3,7,22 low serum calcium concentration, 26-28 and increased intracellular free calcium level. 29,30 The hypothesis can be made that, at least in some cases, the disorders of calcium metabolism and the enhanced vascular resistance with resultant high blood pressure level could both be consequences of a widespread disturbance of cellular calcium handling.
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