Altered Turnover of Polyphosphoinositides in the Erythrocyte Membrane of the Spontaneously Hypertensive Rat

Sophie Koutouzov, Ph.D., Pierre Marche, Ph.D., Arlette Girard, and Philippe Meyer, M.D.

SUMMARY The metabolism of inositol phospholipids of the erythrocyte membrane was compared in normotensive Wistar-Kyoto (WKY), spontaneously hypertensive (SHR), and stroke-prone SHR (SHR-SP) rats. This was performed on isolated ghost membranes by measuring the incorporation of $^{32}$P from $[\gamma-^{32}P]$ adenosine triphosphate (ATP) into the diphosphoinositides (DPI) and the triphosphoinositides (TPI) which were the only $^{32}$P-labeled phospholipids. $^{32}$P-labeling of TPI was altered in adult and 3-week-old SHR as well as in SHR-SP compared to WKY controls; the radioactivity associated with TPI in hypertensive rats was about 30% lower than that associated with TPI in age-matched normotensive controls. By contrast, the radioactivity associated with DPI was similar in both hypertensive and normotensive rats. Measurement of the phosphoinositide distribution in both SHR and WKY indicates that the change observed in $^{32}$P-TPI could not be accounted for by a reduced phosphatidylinositol content in SHR membrane. Measurement of the Mg$^{2+}$-activated TPI-phosphomonoesterase and of the Ca$^{2+}$-activated polyphosphoinositol phosphodiesterase activities did not show any significant difference between SHR and WKY. It thus appears that the altered phosphoinositide metabolism observed in hypertensive rats was a consequence of some alteration in the activity of kinases which are responsible for the conversion of phosphatidylinositol into DPI and TPI. These results also suggest that the defect in phosphoinositide metabolism observed in genetically hypertensive rats was not a consequence of the blood pressure elevation and could be related to the pathogenesis of hypertension. (Hypertension 5: 409-414, 1983)

KEY WORDS • spontaneous hypertension • erythrocyte membrane • phosphoinositide turnover • polyphosphoinositides • phospholipids
with similar magnitude in SHR-SP substrain, and could not be ascribed to difference(s) in phosphatase activities. Together with our previous findings, these results suggest that, in hypertensive animals, alterations occur at the level of the phosphorylation enzymes.

Materials and Methods

Animals

Male rats were used throughout this study. Okamoto spontaneously hypertensive rats (SHR) and age-matched Wistar-Kyoto (WKY) normotensive controls derived from the NIH stock were supplied by Iffa-Credo (France). Unless specified, they were studied at 3 and 12-15 weeks of age. Systolic arterial blood pressure (BP) was recorded by tail sphygmomanometry. Values (in mm Hg, mean ± SEM) were as follows: at 3 weeks, SHR = 115 ± 3, control = 105 ± 3; at 12-15 weeks, SHR = 192 ± 5, control = 130 ± 5.

Spontaneously hypertensive rats of the stroke-prone substrain (SHR-SP) were studied at 7 weeks of age and were compared with 8- to 9-week-old WKY originating from the same Japanese stock. Both SHR-SPs and WKYS were supplied by the Centre d’Eleveage du CNRS (Orléans, France). BP values were as follows: SHR-SP = 170 ± 10; WKY = 120 ± 5 mm Hg.

All animals were given a standard diet with free access to tap water. Blood was sampled by cardiac puncture performed on stunned animals using heparinized syringes and kept at 4°C. It was centrifuged at 800 g for 10 minutes at 4°C, and the red blood cells were washed three times in 154 mM NaCl. The white buffy layer was eliminated by aspiration. On the same day, erythrocyte ghost membranes were prepared according to Schneider and Kirschner. Protein concentration of ghost membranes usually ranged between 3-4 mg/ml as measured by the Lowry et al. procedure. Membrane preparations were stored at -30°C and were used within 2 days.

Incorporation of 32P into Phosphoinositides

This was carried out at 37°C as we have described previously. In 0.5 ml (final volume) of 50 mM Tris-HCl buffer, pH 7.5, 0.3 mg membrane proteins were incubated for 15 minutes in a medium containing 5 mM MgCl2 (unless otherwise specified), 2 mM ATP, and 8-10 μCi [γ-32P] ATP (2000-3000 Ci/mmol; Amersham, England). The reaction was terminated by the addition of the lipid extraction medium (see below).

The influence of Ca2+ ions was studied under similar conditions except that 0.5 mM CaCl2 was present in the incubation medium.

The assay for Mg2+-stimulated TPI-specific phosphomonoesterase was carried out as detailed previously. In brief, 0.6 mg of membrane proteins were 32P-labeled as described above except that the final volume was 1 ml and 15-20 μCi [γ-32P] ATP were used. To stop the reaction, Mg2+ ions and ATP were removed by adding 40 ml of cold Tris-HCl buffer followed by 10 minutes of centrifugation at 48,000 g. This dilution/centrifugation procedure was repeated twice. The resulting pellet, which contained the washed 32P-labeled membranes, was resuspended in 1 ml of cold Tris-HCl buffer and divided into two tubes. Each of these tubes was reincubated for 15 minutes at 37°C in the presence (or absence for controls) of 5 mM MgCl2. Then the reaction was stopped by the addition of the lipid extraction medium (see below).

Materials and Methods

Phospholipid Extraction, Separation, and Analysis

Lipids were extracted from membranes by the addition of 3.75 vol of cold chloroform/methanol/concentrated HCl (20/40/1, vol/vol/vol). The resulting single phase was partitioned by the addition of 1.25 vol each of chloroform and distilled water and centrifuged for 10 minutes at 1500 g. After removal of the upper phase by aspiration, the lower (chloroform) phase was evaporated to dryness under N2, dissolved in chloroform, and stored overnight at -30°C before analysis. Phosphoinositides were separated by one-dimensional thin layer chromatography on cellulose precoated plates (Merck) according to the method of Marche et al. Plates were dried and immersed for 15 minutes in a solution of Nile blue for staining of lipids. Then the areas corresponding to TPI and DPI were scraped off and analyzed either for phosphorous content according to Böttcher et al. or for radioactivity after suspension in 3 ml Unisolve (Koch Light).

Statistical Analysis

Variability is expressed as the mean ± SEM, and tests of significance were performed with the unpaired Student’s t test.

Results

32P-Incorporation into Polyphosphoinositides: Comparison between WKY and Adult SHR, Young SHR, and SHR-SP

It has been reported that incubation of "ghost" erythrocyte membranes with 2 mM ATP (containing [γ-32P] ATP) and 5 mM MgCl2, represents experimental conditions that are close to physiological ones. Therefore, in a first series of experiments, various types of animals originating from the SHR strain were compared with age-matched normotensive WKYS by incubating the isolated ghost membranes under the conditions above. Since these conditions have been reported to promote the 32P-labeling of only polyphosphoinositides (DPI and TPI) among lipids, the results were expressed as the amounts of radioactivity that were incorporated into DPI and TPI.

When 12 to 15-week-old SHRs were compared with age-matched normotensive controls, the radioactivity associated with DPI was similar in both groups of rats whereas that associated with TPI was significantly reduced (30%) in SHR compared to WKY (table 1 A). When experiments were performed on 3-week-old rats, the results were essentially similar to those obtained with adult rats: 32P-DPI was similar in both rat strains whereas 32P-TPI was significantly (22%) lower in SHR than in WKY (table 1 B). Similar experiments
TABLE 1. Incorporation of \(^{32}\)P into Polyposphoinositides of Various Genetically Hypertensive Rats and of Normotensive Controls

<table>
<thead>
<tr>
<th></th>
<th>TPI</th>
<th>DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. 12 to 15-week-old rats</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n = 6)</td>
<td>2.50 ± 0.20</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>SHR (n = 6)</td>
<td>1.75 ± 0.14*</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td>Δ%</td>
<td>-30</td>
<td>0</td>
</tr>
<tr>
<td><strong>B. 3-week-old rats</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n = 8)</td>
<td>2.68 ± 0.23</td>
<td>1.83 ± 0.08</td>
</tr>
<tr>
<td>SHR (n = 8)</td>
<td>2.10 ± 0.17*</td>
<td>1.84 ± 0.08</td>
</tr>
<tr>
<td>Δ%</td>
<td>-22</td>
<td>0</td>
</tr>
<tr>
<td><strong>C. Stroke-prone rats</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n = 7)</td>
<td>3.72 ± 0.14</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>SHR-SP (n = 5)</td>
<td>2.81 ± 0.18†</td>
<td>0.22 ± 0.01†</td>
</tr>
<tr>
<td>Δ%</td>
<td>-24</td>
<td>0</td>
</tr>
</tbody>
</table>

0.3 mg membrane proteins were incubated for 15 minutes in a medium that contained 5 mM MgCl\(_2\) and 2 mM ATP with the label. Results are expressed as \(^{32}\)P nmol incorporated per mg protein per 15 minutes (means ± SE). Numbers in parentheses are the number of experiments, one animal per experiment. Δ% = percent of decrease (—) of \(^{32}\)P incorporation into SHR relative to WKY.

* p < 0.05 compared with WKY.

Overall Influence of Magnesium Concentration

Since the \(^{32}\)P-incorporation into DPI and TPI is under the control of Mg\(^{2+}\)-dependent enzymes, the overall influence of Mg\(^{2+}\) ions on the \(^{32}\)P-labeling of polyphosphoinositides was studied by incubating the membranes with various MgCl\(_2\) concentrations. The results obtained with 10-week-old rats (fig. 2) showed that, whatever the magnesium concentration in the incubation medium (within the range studied), \(^{32}\)P-TPI in SHR was 63% to 70% of the value in WKY, and \(^{32}\)P-DPI was similar in both groups of rats. Figure 2 also shows that, in SHR as in WKY, TPI incorporated more radioactivity than did DPI. This has been already reported in the regular Wistar rat.

Studies of TPI-Specific Phosphomonoesterase Activity

This enzyme whose activity is stimulated by Mg\(^{2+}\) ions has been recently described in the rat erythrocyte where it is membrane-bound. Under conditions in which the membranes and hence polyphosphoinositides are \(^{32}\)P-labeled, the enzyme induces a decrease in...
polyphosphoinositides of SHR and WKY erythrocyte membranes. The 0.3 mg membrane proteins originating from erythrocyte membranes of 10-week-old rats were incubated for 15 minutes in a medium containing 2 mM ATP and MgCl2 at various concentrations. Black symbols = SHR; open symbols = WKY; triangles = radioactivity associated with TPI; circles, radioactivity associated with DPI. Each point is the mean of two experiments.

![Figure 2. Influence of MgCl2 on the 32P-incorporation into polyphosphoinositides of SHR and WKY erythrocyte membranes. The 0.3 mg membrane proteins originating from erythrocyte membranes of 10-week-old rats were incubated for 15 minutes in a medium containing 2 mM ATP and MgCl2 at various concentrations. Black symbols = SHR; open symbols = WKY; triangles = radioactivity associated with TPI; circles, radioactivity associated with DPI. Each point is the mean of two experiments.](image)

Table 2. Effect of Mg2+ on Prelabeled Membranes: Assay for TPI-Specific-Phosphomonoesterase Activity

<table>
<thead>
<tr>
<th>Rat group</th>
<th>TPI</th>
<th>DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 to 15-week-old</td>
<td>73.7 ± 2.4</td>
<td>137.6 ± 3.6</td>
</tr>
<tr>
<td>WKY (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR (6)</td>
<td>74.7 ± 1.9</td>
<td>130.3 ± 2.4</td>
</tr>
<tr>
<td>3 to 4-week-old</td>
<td>58.9 ± 1.8</td>
<td>141.8 ± 5.3</td>
</tr>
<tr>
<td>WKY (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR (12)</td>
<td>57.4 ± 2.6</td>
<td>132.6 ± 4.0</td>
</tr>
</tbody>
</table>

Measurement of the Mg2+-stimulated TPI-phosphomonoesterase activity was performed according to Koutouzov and Marche13 by incubating prelabeled membranes for 15 minutes in a medium containing 5 mM MgCl2 and by measuring the radioactivities associated with TPI and DPI. In controls, the prelabeled membranes were reincubated in the absence of MgCl2. The percentage of radioactivities associated in Mg2+-treated samples to those associated in controls was considered as a measurement of the enzyme activity. Absolute values of controls, taken as 100%, were those mentioned in table 1. Numbers in parentheses indicate the number of experiments, one animal per experiment.

Discussion

Previous investigations have clearly shown that erythrocytes from SHR do not behave as those from normotensive rats with respect to the in vitro incorporation of 32P into polyphosphoinositides.11,12 In addition, recent findings have suggested that, under particular experimental conditions (i.e., 2 mM ATP and 5 mM MgCl2), the polyphosphoinositol turnover in isolated ghost membranes could mimic, at least partially, that in intact red blood cells.12,15,16,19 This prompted us to investigate further the 32P-labeling of polyphosphoinositides of SHR could be superimposed on those of WKY (fig. 3).

Influence of Ca2+ Ions on 32P-Incorporation into Polyphosphoinositides

When membranes were incubated with 2 mM ATP, 5 mM MgCl2, and 10-5 M EDTA, the radioactivities incorporated into TPI and DPI did not differ from those reported above (table 1) in which EDTA was omitted. By contrast, when CaCl2 was present in the incubation medium during the 32P-incorporation into membranes, the radioactivities associated with TPI and DPI decreased concomitantly as a function of the CaCl2 concentration. In both SHR and WKY, 32P-TPI and 32P-DPI appeared to decrease in a parallel manner. In both rat substrains, 0.5–1 10-4 M CaCl2 induced a 25% decrease in the radioactivity associated with both DPI and TPI. In the presence of 10-3 M CaCl2, the radioactivities associated with TPI and with DPI were 30% to 40% of control values (when membranes were incubated in the presence of 10-3 M EDTA). These results clearly showed that in the presence of Ca2+ ions the curves depicting the 32P-labeling of polyphosphoinositides of SHR could be superimposed on those of WKY (fig. 3).
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FIGURE 3. Effect of Ca\(^{2+}\) ions upon the \(^{32}\)P-incorporation into polyphosphoinositides. The 0.3 mg membrane proteins originating from erythrocyte membranes of 12- to 15-week-old rats were incubated in a medium containing 2 mM ATP, 5 mM MgCl\(_2\), and CaCl\(_2\) at various concentrations. Results are expressed as the percentage of radioactivity incorporated into controls, i.e., when the incubation medium contained 10\(^{-5}\) M EDTA instead of CaCl\(_2\). Results are means ± se, from two to four experiments, one animal per experiment.

blood pressure. Several membrane alterations have been described in both SHR and SHR-SP when compared to WKY.\(^2\) Our finding that hypertensive rats of the SHR-SP substrain behaved as SHRs with respect to \(^{32}\)P-labeling of TPI was therefore not unexpected. It lends support to the hypothesis that membrane changes noted in erythrocytes may be related to the pathogenesis of hypertension.

We previously reported that TPI labeling did not occur solely via the phosphorylation of the pre-existing endogenous pool of DPI but was probably the result of the successive reactions of PI-kinase and DPI-kinase.\(^19\) This prompted us to investigate the possibility that the decreased \(^{32}\)P-labeling of TPI in SHR was due to a reduced PI content of the membrane. Indeed, in adult animals, the PI content of the erythrocyte membrane of SHR was significantly lower than that in WKY, as has been published by Boriskina et al.\(^10\) By contrast, in 3 week-old animals, PI (and DPI) contents did not differ significantly in SHR and in WKY membranes. Thus, the results obtained with young rats did not support the above hypothesis.

Whatever the Mg\(^{2+}\) concentration of the incubation medium (up to 10 mM), it was clear that \(^{32}\)P-TPI in SHR membranes was only 70% of \(^{32}\)P-TPI in WKY membranes whereas \(^{32}\)P-DPI was similar in both rat strains. The presence of Mg\(^{2+}\) ions has been demonstrated to be necessary for the activation of both PI- and DPI-kinases as well as of TPI-specific phosphomonoesterase.\(^12\)-\(^15\) Since the \(^{32}\)P-labeling of TPI results from the balance between these enzyme activities, the reduced amount of radioactivity incorporated into TPI of SHR compared to WKY could therefore be a consequence of alteration(s) in one or several of these enzymes. In this context, our results obtained with prelabeled membranes (table 2) show that the activity of TPI-phosphomonoesterase in SHR did not differ from that in WKY.

In the experiments described above, membranes were prepared in the presence of EDTA and were thereafter processed in Ca\(^{2+}\)-free media. Thus, under these conditions, it appears that the only enzymic activities that participate in the phosphoinositide metabolism of isolated membranes were PI-kinase, DPI-kinase, and TPI-phosphomonoesterase. By contrast, when intact erythrocytes were incubated in a medium that contained 2 mM CaCl\(_2\),\(^12\) the additional participation of the Ca\(^{2+}\)-activated polyphosphodiesterase to the phosphoinositide metabolism could not be ruled out. Indeed, such a phospholipase C-like activity has been demonstrated in the erythrocyte where the enzyme is membrane-bound and capable of attacking both DPI and TPI with equal facility.\(^12\)-\(^15\) A difference in this enzyme activity in SHR compared to WKY could also explain the differences in \(^{32}\)P-TPI and \(^{32}\)P-DPI that we have observed when intact erythrocytes were incubated with \(^{32}\)P-orthophosphate.\(^11\) Measurements of the Ca\(^{2+}\)-activated polyphosphoinositide phosphodiesterase activity in isolated membranes from SHR and WKY showed that, irrespective of Ca\(^{2+}\) concentration, the membranes of both groups of rats behaved similarly. If one assumes that the membrane-bound activity of this enzyme in isolated membrane reflects its activity in the intact cell, our data indicate that the differences observed between intact erythrocytes of SHR and those of WKY\(^12\) could not be ascribed to differences in activity of polyphosphoinositide phosphodiesterase.

From these findings, PI-kinase and/or DPI-kinase appear to be the best candidate(s) to account for the difference in the phosphoinositide metabolism between SHR and WKY. Previous experiments performed on isolated membranes partially solubilized by Triton \(\times 100\) suggested that PI-kinase activity was similar in membranes of both SHR and WKY\(^12\) and led to the hypothesis that only the DPI-kinase activity was lowered in SHR. In SHR, this would result in both a decrease in \(^{32}\)P-TPI and an increase in \(^{32}\)P-DPI, a result
that has been reported from experiments using intact erythrocytes. Comparison of data obtained in intact cells with those obtained in isolated ghost membranes in which only the decrease in 32P-TPI in SHR could be observed suggests that, within the SHR erythrocyte membrane/cytosol interactions may occur that can modify phosphoinositide metabolism. This is supported by recent reports describing structural membrane alterations within the erythrocyte of SHR, and in various cell types. The possible relationship between these changes and that of phosphoinositide metabolism has been already discussed. It remains to be established that an impaired metabolism of inositol lipids is diffused to other tissues of genetically hypertensive rats and in particular to excitable cells. Likewise, the precise genetic link between the membrane defect and the transmission of hypertension remains to be specified. Nevertheless, the present study provides additional evidence that erythrocyte membranes of spontaneously hypertensive rats exhibit inherited characteristics that may be related to the pathogenesis of hypertension.

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