Glomerular Basement Membrane Polyanion Distribution and Nitric Oxide in Spontaneous Hypertensive Rats: Effects of Salt Loading and Antihypertensive Therapy with Propranolol

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Cationic colloidal gold (CCG), a polycationic histochemical probe, was used to analyze the distribution of glomerular basement membrane (GBM) polyanions, mainly heparan sulfate proteoglycan in spontaneous hypertensive rats (SHR) with or without salt loading and antihypertensive treatment with propranolol. The changes of mean GBM width and anionic sites distribution were assessed by electron microscopy. Plasma and urinary nitrates (NOx) were measured by nitrite (NO2) + nitrate (NO3), stable metabolites of NO. SHR had decreased NO production and increased GBM width (27%) compared with the control Wistar-Kyoto (WKY) rats. The chronic high dietary salt intake resulted in a significant increase in blood pressure, proteinuria, and renal function in the SHR rats. The chronic high salt dietary intake resulted in a decrease in NO in the WKY and a further reduction in NO production in the SHR. The GBM anionic sites count was similar in the SHR and WKY nonsalt-loaded groups, 13.5 ± 0.5 and 12.8 ± 0.4 CCG counts/μm GBM, respectively, but significantly lower in both salt-loaded SHR and WKY, 9.9 ± 0.55 (P < .01) and 9.6 ± 0.55 (P < .01) CCG counts/μm GBM, respectively. Antihypertensive treatment with propranolol in the salt-loaded SHR group resulted in lower blood pressure, a further decrease in NO production, but no significant changes in GBM width and anionic sites count. It is concluded that chronic high salt intake may be deleterious to the permselectivity of the GBM. A low NO production state that results from chronic salt loading in already hypertensive rats will result in more severe organ (renal) damage, most probably by the addition of the loss of GBM permselectivity to the existing pathomorphologic changes. Am J Hypertens 2000;13:838–845 © 2000 American Journal of Hypertension, Ltd.

KEY WORDS: Cationic colloidal gold, glomerular basement membrane anionic sites, glomerular basement membrane width, nitric oxide, saline loading, spontaneous hypertensive rats.
Chronic oral sodium chloride loading in spontaneous hypertensive rats (SHR) results in very severe hypertension, decreased glomerular filtration rate, proteinuria and glomerulosclerosis.¹

Heparan sulfate proteoglycans (HSPG) are major components of the glomerular basement membrane (GBM),²,³ and play a key role in the molecular organization and function⁴ of the basement membrane. Their presence is essential for maintenance of the charge-dependent selective permeability of the GBM.⁵–⁹. Loss of GBM anionic sites has been described in experimental diabetic nephropathy,¹⁰–¹⁴ amino-nucleoside nephropathy,¹⁵–¹⁸ and other nephropathies.¹⁹–²⁴

Heintz et al²⁵ found increased excretion into the urine of GBM-specific heparan sulfate proteoglycans in essential hypertension. These findings, however, do not provide direct evidence regarding the distribution of the GBM polyanion. In experimental hypertension caused by subtotal nephrectomy, Dubrulle et al²⁶ showed thickening of the GBM, decreased anionic sites density, and proteinuria. A significant decrease in the proteoglycan synthesis was found in the arteries of 28-week-old Wistar-Kyoto (WKY) and SHR rats; however, the SHR carotid arteries showed an increased synthesis of proteoglycans.²⁷ We found that chronic salt loading in salt-sensitive hypertensive rats was associated with significant decrease in GBM anionic sites mainly HSPG.²⁸ The L-arginine–nitric oxide synthase (NO system) was extensively studied in both experimental and human hypertension.²⁹–³⁵ An impaired NO response was considered as caused by endothelial dysfunction.³⁶–⁴⁸ The NO system seems to react differently in salt-sensitive and SHR rats.³⁴,⁴³–⁴⁵

The aim of the present study was to determine the distribution of the GBM anionic sites in SHR, with or without chronic salt loading and antihypertensive therapy with propranolol. Analysis of the GBM morphology and of the cationic colloidal gold (CCG) labeling densities on different regions of the GBM was performed by electron microscopy. This marker was shown to provide a useful postembedding histochemical probe for the localization and evaluation of sulfated proteoglycans in cell membranes⁴⁹–⁵¹ and secretory granules,⁵² as well as extracellular matrix constituents, including GBM in normal and nephrotic kidneys.²¹,⁵³

A possible relationship between GBM changes and nitric oxide system was hypothesized. Nitrite + nitrate (NO₂⁻ + NO₃⁻ = NO₃⁻) stable metabolites of nitric oxide were measured in the plasma and urine.

**MATERIAL AND METHODS**

Spontaneous hypertensive rats and WKY controls (180 to 200 g) both sexes, 8 weeks old, were used in all experiments. After an acclimatization period of 3 days in individual metabolic cages (free access to normal rat chow and water) a 24-h urine collection was obtained and a blood sample was drawn under general anesthesia (Ketalar, 70 mg/kg body weight, and Xylocain, 6 mg/kg body weight intraperitoneally). The blood samples and the 24-h urine collections were stored at −20°C for laboratory determinations and taken as basal values. Immediately after the blood sampling, the rats were randomly allocated to one of five experimental groups: group 1 (SHR, n = 15), SHR rats had free access to normal rat chow and tap water throughout the experiment; group 2 (SHR + salt loading [S], n = 15), same as group 1 but the rats had 8% NaCl in the food for 1 month; group 3 (SHR + S + propranolol [Pro], n = 15), same as group 2 but the rats received 30 mg/L of propranolol in their drinking water; group 4 (WKY, n = 15), control WKY rats had free access to normal rat chow and water throughout the experiment; and group 5 (WKY + S, n = 15), same as group 4 but the rats had 8% NaCl in the food for 1 month.

At the end of the experiments, 24-h urine collections in the individual metabolic cages, the rats were sacrificed under general anesthesia and final blood samples were obtained. The kidneys were rapidly taken out, cortical slices were immediately placed in Karnovsky fixative, pH 7.4, for histochemical studies and in glutaraldehyde with OsO₄ for GBM morphology studies.

**Laboratory Determinations** Levels of blood urea nitrogen, plasma, and urine creatinine, sodium, potassium, and urine total proteins were determined by standard laboratory techniques. Plasma NOₓ and urine NOₓ (nitrate and nitrite) concentrations were determined as previously described.²⁴,⁵⁵ After the reduction of NO₃⁻ to NO₂⁻ by a 90-min incubation in a tilting bath (37°C) using nitrate reductase from Escherichia coli (prepared in our laboratory) and NADPH (Sigma, St. Louis, MO) as cofactor, NO₂⁻ was determined with Griess reaction. The plasma NOₓ is given in micromoles per liter, the urine NOₓ is expressed in micromoles per day per 100 grams of body weight. Creatinine clearance (in microliters per minute per 100 grams of body weight), urinary protein (in milligrams per day per 100 grams of body weight), and urinary sodium and potassium (in microequivalents per day per 100 grams of body weight) were calculated.

**Tissue Fixation and Embedding** Tissue blocks (1 mm³) of Karnovsky’s fixed kidney cortical slices were washed overnight with phosphate-buffered saline (PBS), dehydrated in ethanol, and embedded in LR-white (Polyscience, Washington, PA). For electron microscopic morphology, similar tissue blocks were postfixed with 1% OsO₄ in sodium cacodylate buffer,
**TABLE 1. BLOOD PRESSURE AND BIOCHEMISTRY IN THE DIFFERENT EXPERIMENTAL GROUPS AT THE END OF THE STUDY**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>SHR</th>
<th>SHR + S</th>
<th>SHR + S + Pro</th>
<th>WKY</th>
<th>WKY + S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mm Hg)</td>
<td>170 ± 8</td>
<td>197 ± 4.4*</td>
<td>168 ± 4†</td>
<td>98 ± 4‡</td>
<td>112 ± 6‡</td>
</tr>
<tr>
<td>BUN (mg %)</td>
<td>17.7 ± 0.6</td>
<td>15.7 ± 1.4</td>
<td>14 ± 0.9</td>
<td>20.3 ± 2.2</td>
<td>16.7 ± 4.4</td>
</tr>
<tr>
<td>C r (µL/min/100 g BW)</td>
<td>572 ± 48</td>
<td>408 ± 53*</td>
<td>504 ± 64‡</td>
<td>568 ± 37</td>
<td>539 ± 23‡</td>
</tr>
<tr>
<td>P-K (mEq/L)</td>
<td>4.9 ± 0.2</td>
<td>4.7 ± 0.5</td>
<td>4.8 ± 0.5</td>
<td>4.3 ± 0.3</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>P-Na (mEq/L)</td>
<td>138 ± 1</td>
<td>132 ± 7</td>
<td>135 ± 1.7</td>
<td>131 ± 13</td>
<td>137 ± 1</td>
</tr>
<tr>
<td>Uprot (mg/day/100 g BW)</td>
<td>39 ± 7</td>
<td>138 ± 21*</td>
<td>147 ± 31</td>
<td>8.5 ± 1.3‡</td>
<td>23.5 ± 5.1‡</td>
</tr>
<tr>
<td>U-Na (µEq/day/100 g BW)</td>
<td>200 ± 44</td>
<td>1860 ± 90*</td>
<td>2270 ± 146‡</td>
<td>245 ± 78</td>
<td>1810 ± 290*</td>
</tr>
<tr>
<td>U-K (µEq/day/100 g BW)</td>
<td>228 ± 35</td>
<td>647 ± 65*</td>
<td>399 ± 16‡</td>
<td>644 ± 12‡</td>
<td>799 ± 31‡</td>
</tr>
<tr>
<td>PNOx (µM/L)</td>
<td>20.30 ± 1.36</td>
<td>16.82 ± 1.03*</td>
<td>13.86 ± 1.10‡</td>
<td>20.13 ± 3.25</td>
<td>13.07 ± 0.83‡</td>
</tr>
<tr>
<td>UNOx (µmol/day/100 g BW)</td>
<td>1.37 ± 0.179</td>
<td>1.17 ± 0.234</td>
<td>0.852 ± 0.242*</td>
<td>2.215 ± 0.242‡</td>
<td>1.412 ± 0.123*</td>
</tr>
</tbody>
</table>

* P < .05 (at least) v the respective untreated groups; † P < .05 (at least) v SHR + S group; ‡ P < .05 (at least) WKY v untreated SHR group.

**pH** 7.4, for 1 h at 4°C, washed for 1 h in the same buffer, dehydrated in ethanol and propylene oxide, and embedded in Araldite (Polysciences).

**Electron Microscopy** For electron microscopic morphology, ultrathin alaridate sections were mounted on naked 400 mesh grids, stained with uranyl acetate and lead citrate, and coated with carbon. For electron microscopic histochemistry, ultrathin LR-white sections of approximately 60 nm were mounted on 200 mesh nickel grids, coated with Formvar films impregnated with carbon. CCG was prepared by the stabilization of colloidal gold, 12 nm average diameter, with poly-L-lysine (molecular mass, 27,000). The sections were treated with 1% bovine serum albumin in PBS for 5 min, washed with PBS, labeled with CCG, diluted by 1:20 (the ratio of volumes CCG:PBS) in PBS for 1 h, rinsed with a stream of distilled water, and stained for 15 min with saturated uranyl acetate in 50% ethanol. The examination of all sections was carried out with a JEM-100B (JEOL, Tokyo, Japan) electron microscope at 80 KV.

**Morphometry** Electron micrographs (original magnification, ×18,000) were obtained. The thickness of the GBM was estimated by determining the harmonic mean of a series of orthogonal intercept measurements. The GBM thickness was considered as the distance between the endothelial and epithelial cell membranes opposed to the GBM without consideration of the clarity of the cell membranes profiles (ie, tangential cuts). Segments of GBM derived from at least eightglomeruli of each rat of each group were used for analyses.

Analyses of the labeling densities of the gold particles attached to different regions of the GBM were performed on electron micrographs of LR-white sections at an original magnification of 25,000. The length of GBM was measured and the number of gold particles attached to the lamina rara interna (LRI) and the lamina rara externa (LRE) regions of the GBM were counted separately with a MOP videoplan morphometric system (Kontron, Eching/Munchen, Germany). The Kontron standard program carried out the labeling density calculation.

**Blood Pressure Measurement** Systolic blood pressure was measured in awake rats by a tail-cuff method (Harvard Instruments, Harvard Apparatus United, Edenbridge, KY) and recorded on a double channel recorder. Blood pressure was determined in all rats at the end of the experiment.

**Statistical Analysis** Data is given as mean ± SD. Student t test was used to assess significance. P < .05 was considered significant.

**RESULTS**

**Blood Pressure and Renal Function in the Experimental Groups** Four weeks of high oral salt intake added to the food in the SHR resulted in very high blood pressure (197 ± 4.4 mm Hg compared with 112 ± 6 mm Hg, P < .001, in the respective WKY rats). The salt-loaded SHR group, which was concurrently treated with propranolol, had a decreased blood pressure (168 ± 4 mm Hg, P < .01) compared with untreated, salt-loaded SHR rats (Table 1 and Fig. 1).

The creatinine clearance decreased in the salt-loaded SHR group compared with the untreated SHR group. The administration of propranolol prevented partially this reduction in creatinine clearance. The sodium and potassium excretion lower in the propranolol-treated animals, but the sodium excretion was higher and potassium excretion increased as expected in the salt-loaded groups, but the sodium excretion was higher and potassium excretion lower in the propranolol-treated animals. The daily urine protein excretion rate was 39.7 ± 8 mg/day/100 g body weight in SHR and the respective WKY rats, 23.5 ± 5.1 mg/day/100 g body weight in SHR and the respective WKY rats, 23.5 ± 5.1...
mg/day/100 g body weight ($P < .001$). The propranolol did not affect proteinuria.

Changes in NO$_2$ + NO$_3$  The plasma value of NO$_2$ + NO$_3$ (PNO$_x$) in WKY or SHR untreated rats were similar, 20.13 ± 3.25 and 20.3 ± 1.36 μmol/L, respectively. Salt loading resulted in a significant decrease of PNO$_x$ in both WKY + S and SHR + S, 13.07 ± 0.83 and 16.82 ± 1.03 μmol/L, respectively, compared with the respective nonsalt-loaded groups. The PNO$_x$ in the SHR + S group orally supplemented with propranolol was significantly lower, 13.86 ± 1.1 μmol/L compared with the group not treated with propranolol ($P < .001$). The urinary NO$_2$ + NO$_3$ (UNO$_x$) in the control WKY was significantly higher compared with the untreated SHR, 2.215 ± 0.242 and 1.370 ± 0.179 μmol/day/100 g body weight ($P < .001$), respectively. The salt loading in WKY rats resulted in a 60% reduction of UNO$_x$ level compared with the values of nonsalt-loaded WKY rats. The propranolol treatment resulted in the lowest UNO$_x$ compared to all other groups at the end of the study (Table 1).

**Morphologic Changes in the GBM** Without any treatment, the average width of the GBM of the SHR (430 ± 130 nm) was higher by 30% than that of the WKY rats (330 ± 40 nm). After 1 month of high salt intake the average width of the GBM in the SHR and WKY groups was 460 ± 120 nm and 340 ± 40 nm, respectively. Propranolol administration also did not influence the average GBM width (Table 2 and Figs. 1 and 2).

**Binding of CCG to the GBM** Analysis of binding CCG to the GBM was carried out on thin LR-white sections, at a magnification of 25,000. In all samples studies, the distribution of binding of CCG to GBM was highly asymmetrical, exhibiting considerably high (approximately twofold) density of attached gold particles in the LRE portion of the GBM as compared with the LRI. Morphometric analysis of the overall (LRE + LRI) density of CCG particles in the different GBM of the different groups indicate that without sodium loading, the average binding densities in the WKY and SHR groups are similar. But, after 4 weeks of sodium loading, in both the WKY + S and SHR + S, the average CCG binding to the GBM was considerably decreased, approximately by 26% in both groups. The reduction in CCG binding was not proportionally similar in the LRE and LRI regions of the GBM, about 12% reduction of CCG binding in the LRE and about 50% reduction in the LRI (Table 2 and Figs. 1 and 3). Propranolol treatment did not influence the CCG count on the different GBM portions.

**DISCUSSION**

Hypertension is complex, polygenic, and can be multifactorial. Among the factors that had been identified as predisposing to hypertension are genetic factors. SHR rats represent an experimental model of genetic hypertension.$^{59}$ The kidney is a major target organ for hypertension-induced damage. Most of the primary and secondary chronic renal diseases are also associated with secondary hypertension, proteinuria, and further renal damage, nephrosclerosis. Heparan sulfate proteoglycans
are major components of the GBM and are essential for the maintenance of the selective permeability. Loss of anionic sites provided by HSPG is associated with a number of nephropathies. Loss of HSPG might occur as the result of reduced synthesis, enhanced turnover, or degradation of HSPG. However, the exact mechanism of this process is not well understood.

The present work demonstrated that chronic salt loading resulted in a significant increase in blood pressure and protein excretion in SHR and much less in WKY rats. This was associated with a significant decrease in the creatinine clearance of the salt-loaded SHR group. As expected urinary sodium excretion increased in salt-loading experiments. The administration of propranolol results in a lower blood pressure.

The GBM morphology revealed significant differences between SHR and WKY rats without salt loading. The SHR rats are characterized by a much greater GBM width; almost 50% of the GBM had a width of more than 400 nm. The salt loading, however, did not result in any further changes in GBM width, either in the SHR or the WKY rats. Also antihypertensive therapy did not change the GBM morphology in SHR rats. As previously suggested by us and other investigators, pathophysiologic changes of the GBM (ie, increased width and decreased membrane permselectivity) are pathogenic mechanisms for proteinuria. In the early onset of preeclampsia, a strong correlation was found between the number of GBM anionic sites and the severity of proteinuria. This was also associated with increased GBM width in 80% of the cases. It is interesting to note that a threefold increase in urinary protein excretion was present in all salt-loaded rats compared with their respective nonsalt-loaded groups and this was associated with a similar decrease in GBM anionic sites. It can be speculated that the higher protein excretion in the untreated SHR com-

**TABLE 2. GBM WIDTH AND ANIONIC SITES COUNT IN THE DIFFERENT EXPERIMENTAL GROUPS AT THE END OF THE STUDY**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Average Width (nm) ± SD</th>
<th>LRE Count/μm GBM</th>
<th>LRI Count/μm GBM</th>
<th>CCG Counts/μmGBM ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>430 ± 33</td>
<td>8.9</td>
<td>4.4</td>
<td>13.3 ± 0.46</td>
</tr>
<tr>
<td>SHR + S</td>
<td>460 ± 31</td>
<td>7.8</td>
<td>2.1</td>
<td>9.9 ± 0.55*</td>
</tr>
<tr>
<td>SHR + S + Pro</td>
<td>440 ± 29</td>
<td>7.6</td>
<td>2.1</td>
<td>9.7 ± 0.77*</td>
</tr>
<tr>
<td>WKY</td>
<td>330 ± 10†</td>
<td>9</td>
<td>4.5</td>
<td>13.5 ± 0.50</td>
</tr>
<tr>
<td>WKY + S</td>
<td>340 ± 11†</td>
<td>7.8</td>
<td>2.1</td>
<td>9.3 ± 0.55*</td>
</tr>
</tbody>
</table>

* P < .01 (at least) v the respective untreated groups; † P < .01 (at least) v the respective SHR groups.

![FIG. 2. Electron micrographs of araldite sections of kidneys. (End of study). SHR rat (A), WKY rat (B), SHR + S (C), WKY + S (D), SHR + S + propranolol (E). (original magnification 18,000.)](image1)

![FIG. 3. Electron micrographs of LR-white sections of kidneys. SHR rat (A1), WKY rat (B1), SHR + S (C1), WKY + S (D1), SHR + S + propranolol (E1) labeled with CCG. (original magnification 25,000.)](image2)
pared with the respective WKY was caused by the changes in GBM width and the proteinuria that followed salt loading resulted from changes in GBM anionic sites/permselectivity.

Recent publications of Raij and co-workers determined that the administration of salt to salt-sensitive rats resulted in a decrease in NO production and an impaired endothelial function compared with salt-resistant hypertensive rats. These factors were also present at the molecular level in different tissues. The SHR rats were found to have an increased expression of the cNOS isoform in the kidney and aorta compared with the control WKY rats. However, these were similar to the values in salt-sensitive rats before salt loading. It was suggested that NOS and NO expression increases in the tissues of SHR in response to hypertension. A similar high blood pressure increase in salt-loaded salt-sensitive rats and SHR resulted in much more kidney and heart damage.

Our results show that salt loading in SHR rats resulted in a significant decrease in NO production as shown by the UNOx and PNOx results compared with control WKY salt-loaded rats. However, a decrease in NO was found also in the WKY salt-loaded rats. It is interesting that chronic salt loading changed the functional barrier of the GBM. The GBM anionic sites decreased significantly in salt-loaded SHR and WKY rats. This effect of salt loading on the reduction of the GBM charge/permselectivity and proteinuria were also found in salt-sensitive hypertensive rats and in adriamycin-induced nephropathy, both characterized by less NO production. This suggests that a lower NO production state, as a result of chronic salt loading in already hypertensive rats, will result in more severe organ (renal) damage. Most probably in addition to the loss of GBM permselectivity from the existing pathomorphologic changes. In SHR rats, the addition of high salt intake tended to inhibit the response to acetylcholine. With impaired nitric oxide synthesis the coronary and renal systems of SHR are more vulnerable and the end organ damage is worsened by an increase in salt intake.

Propranolol decreased the blood pressure and also partially prevented the decrease in renal function. However, these effects were not accompanied by improvement/normalization of GBM or anionic sites distribution changes caused by salt loading. The angiotensin converting enzyme inhibition of captopril was found to have a beneficial effect on the decrease in GBM anionic sites in SHR with streptozocin-induced diabetes. We found that the beneficial effect of captopril on the progression of chronic renal failure in 5/6 nephrectomized rats (better function and less proteinuria) was caused by the modulation of the NO system. We also found that the L-arginine supplementation in adriamycin-induced nephropathy resulted in an almost normalization of GBM anionic sites and less proteinuria.

All these results support the hypothesis that dietary salt loading and decreased NO production result in important changes in GBM permselectivity, proteinuria, and renal failure. It remains to be established whether new β-blockers such as carvedilol, calcium channel blockers, or angiotensin type 1 receptor blockers have a beneficial effect in salt sensitive or SHR salt loading-induced severe hypertension.

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