Effects of Deoxycorticosterone-Salt Hypertension on Cell Ploidy in the Rat Aorta

Alice H. Lichtenstein, Peter Brecher, and Aram V. Chobanian

SUMMARY We investigated the effect of deoxycorticosterone-salt hypertension and its reversal on nuclear ploidy in rat aortic smooth muscle cells, using flow cytometry. Systolic blood pressure and the percentage of cells with tetraploid nuclei increased over a 4-week period and then remained constant. In control rats, 6.0 ± 0.6% of aortic cells had tetraploid nuclei, and this increased to 27.0 ± 1.1 and 26.6 ± 2.4% after 4 and 8 weeks of deoxycorticosterone-salt treatment, respectively. Computer analysis of the forward light scatter data obtained from the cell sorter indicated that the average tetraploid cell was approximately 1.6-fold larger than the average diploid cell in both normotensive and hypertensive animals. Analysis of the DNA content of intact cells and isolated nuclei indicated that the increased DNA content per cell was predominantly attributable to tetraploid nuclei rather than binucleated cells. Reversal of hypertension with either a low sodium diet for 8 to 13 weeks or chlorothiazide administration failed to reverse the abnormalities seen in arterial smooth muscle cell ploidy, despite normalization of the blood pressure and the ratio of heart to body weight.

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KEY WORDS • smooth muscle cell • hypertrophy • polyploidy • artery • deoxycorticosterone-salt hypertension

INCREASED vascular mass is a well-documented response of large arteries to experimental hypertension, yet the biochemical events underlying this phenomenon remain unclear. Several recent studies have demonstrated that smooth muscle cell hypertrophy is a major factor responsible for the increased mass of smooth muscle occurring in spontaneously hypertensive rats (SHR) or two-kidney, one clip Goldblatt hypertensive rats. The hypertrophy noted in both animal models appeared to be due in part to a marked increase in cells with polyploid nuclei; the number of these cells was proportional to both the duration and extent of hypertension. Antihypertensive drug therapy prevented a further increase in cells with polyploid nuclei that normally developed in SHR between 3 and 7 months of age, but therapy did not reduce the number already present in vascular tissue.

The studies cited above indicate that cells with polyploid nuclei are an important cell type in arterial tissue from hypertensive rats. In previous studies, we have shown that hypertension produces several chemical and enzymatic changes in rat aortic tissue, and those changes are influenced differentially by reversal of the hypertension. To our knowledge, there are no biochemical data on the vascular smooth muscle cell with a tetraploid nucleus. The ability to distinguish between the biochemical properties of diploid and tetraploid vascular cells will clearly be important for future research. In the present study, we have examined the ploidy of aortic medial cells from animals in which hypertension was induced by deoxycorticosterone (DOC)-salt treatment and was reversed by dietary sodium restriction with or without diuretic administration. Our findings indicate that in DOC-salt-treated rats, increased polyploidy of aortic smooth muscle cells occurs even with periods of DOC-salt administration as brief as 2 weeks. These changes in ploidy are not reversed by long-term normalization of blood pressure.

Methods Deoxycorticosterone-Salt Treatment

Groups of 9-week-old male Wistar rats (Charles River Breeding Laboratory, Wilmington, MA, USA) were uninephrectomized under sodium pentobarbital anesthesia (50 mg/kg; Abbott Laboratories, North Chicago, IL, USA). After a 1-week postsurgical recovery period, the rats were treated twice weekly with deoxycorticosterone pivalate (Percoten; Ciba, Summit, NJ, 02118).
Systolic blood pressure was monitored by tail cuff plethysmography and a photoelectric cell detector.³

**Smooth Muscle Cell Suspensions**

Rats were killed with an overdose of sodium pentobarbital after the designated treatment period. Cell suspensions were prepared by a modification of the methods of Ives et al.⁴ The aorta was removed from the arch to the iliac bifurcation and split longitudinally. The media was dissected free of adventitia and placed in a modified Hanks’s balanced salt solution containing 0.2 mM CaCl₂, pH 7.4. The tissue was cut into 5-mm² pieces with a Mcllwain Tissue Chopper (Brinkmann Instruments, Westbury, NY, USA) and incubated in 5 ml of Hanks’s buffer containing 10 mM glucose, 12.5 mM hydroxyethylpiperezinesulfonic acid, 750 U of collagenase (Type 1; Sigma, St. Louis, MO, USA), 20 U of elastase (Type II, Sigma), 3 mg of lima bean trypsin inhibitor (Sigma), 100 U of deoxyribonuclease (Type 1; Cooper Biomedical, Malvern, PA, USA), and 1% bovine serum albumin (Fraction V, Sigma). The tissue was incubated at 37°C in a shaking incubator for 75 to 90 minutes, with the exact length of time determined by visual assessment of the turbidity of each sample. The incubation was stopped by adding 20 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 200,000 U of penicillin, 0.2 g of streptomycin, and 0.5 mg of Fungizone (GIBCO, Chagrin Falls, OH, USA). The residual tissue was dispersed by aspirating it 20 times through a 13-gauge needle, and the cells were harvested by passing the mixture through a 105-μm nylon mesh. The material remaining on the mesh was reincubated with fresh buffer containing the enzymes for 45 to 60 minutes and treated as described above. The resulting cells from the two incubations were pooled and harvested by centrifugation at 100 g for 10 minutes. Approximately 95% of the cells were viable as assessed by their ability to exclude trypan blue.

**Determination of Cell Ploidy**

Ploidy was determined both in whole cells and in isolated nuclei with flow cytometry. For determination of ploidy in whole cells, an aliquot of the cell suspension was fixed with 70% ethanol for 30 minutes; the cells were centrifuged at 100 g for 10 minutes, resuspended in phosphate-buffered saline containing ribonuclease (1 mg/ml, Sigma), and incubated for 30 minutes at 23 to 25°C. The cells were then centrifuged again and resuspended in a solution containing 75μM propidium iodide (Sigma) and 3.4 mM sodium citrate. For determination of ploidy in isolated nuclei, the cell suspension was centrifuged at 100 g for 10 minutes and resuspended in a solution containing 0.1% Triton X-100, 75 μM propidium iodide, and 3.4 mM sodium citrate. Visual inspection of the preparation showed a predominance of nuclei and an absence of intact cells. All preparations were filtered through a 37-μm nylon mesh just before analysis by flow cytometry.

Fluorescence and forward light scatter (2-15 degrees) were determined with a FACS-IV dual laser system (Becton-Dickinson, Sunnyvale, CA, USA) interfaced with a PDP 11/23 computer (Digital, Maynard, MA, USA). The suspension was excited at 488 nm with an argon-ion laser (164-05, Spectro Physics, Mountain View, CA, USA), and the emission was measured at 625/35 nm. In most cases 10⁴ cells or nuclei were analyzed for each sample.

Significant differences in ploidy between the control and treated groups were determined by a two-way analysis of variance.

**Results**

Preliminary studies were initiated to determine whether rats treated with DOC-salt had an increase in the proportion of arterial smooth muscle cells with polyploid nuclei. Figure 1 shows representative flow cytometric tracings of intact cells from a control rat (see Figure 1A) and a hypertensive rat treated with DOC-salt for 4 weeks (see Figure 1B). The data show the distribution of cells as a function of the amount of DNA contained in the cells. In both samples two cell populations were found, one population having twice as much DNA as the other. In the control tissue, 93%
of the cells were diploid and 7% were tetraploid. A fourfold increase in tetraploid cells was found as a result of DOC-salt treatment (see Figure 1B). Furthermore, relatively few cells had intermediate amounts of DNA, suggesting that relatively few cells were in the S phase of the cell cycle. No evidence of cells with octaploid nuclei was found.

Figure 2 shows a computer-generated plot of fluorescence and forward light scatter for a representative intact cell preparation from a control and a hypertensive animal. Tetraploid cells from both the normotensive and hypertensive animals were larger than the corresponding diploid cells. Analysis of the data indicated that the ratio of the average size of the tetraploid cells to the average size of the diploid cells was 1.67 ± 0.05 and 1.61 ± 0.06 (mean ± SE) for six hypertensive and seven normotensive rats, respectively.

Since the change in the percentage of arterial smooth muscle cells with tetraploid nuclei in response to hypertension appeared to occur quickly, we next investigated the time course of the response. Rats were made hypertensive with DOC-salt for periods ranging from 1 to 8 weeks. As shown in Figure 3A, systolic blood pressure increased over a 4-week period, reaching a maximum of about 175 mm Hg. Treatment with DOC-salt for 4 additional weeks did not affect pressure appreciably. The increase in the percentage of tetraploid nuclei also reached a maximum at about 4 weeks (Figure 3B) and occurred at a rate that was similar to that for blood pressure.

The relationship between blood pressure and tetraploidy for a large group of DOC-salt–treated animals is presented in Figure 4 (opposite). The animals were treated with DOC-salt for periods ranging from 1 to 8 weeks. With increasing blood pressure, there was a steady increase in the proportion of cells with tetraploid nuclei. Analyses of the data indicated a significant correlation between the two parameters (r = 0.766, p < 0.001).

It is possible that the cell population referred to as having tetraploid nuclei actually contained cells with two diploid nuclei. In our studies we employed two
methods to assess DNA content, using either intact cells or isolated nuclei stained with fluorescent dye. A comparison of the data obtained using both methods in 45 DOC-salt-treated rats is shown in Figure 5. Good agreement exists ($r = 0.91, p < 0.001$) between the results obtained with the two procedures. Thus, there is strong evidence for the presence of a single nucleus with twice the DNA content of a diploid nucleus.

We also investigated the effect of hypertension reversal on ploidy. Table 1 summarizes the results from a series of experiments in which rats were treated with DOC-salt for 4 weeks and then subjected to three different regimens for reversing the hypertension. Control groups were included for each treatment. After treatment with DOC-salt, systolic blood pressure and the percentage of cells with tetraploid nuclei increased to 207 mm Hg and 27%, respectively. After 8 or 13 weeks of a low sodium diet, blood pressure was effectively reduced to normotensive levels, but the percentage of cells with tetraploid nuclei remained essentially unchanged and well above control levels. Along with the lowering of blood pressure, the ratio of heart to body weight returned to normal (3.98 vs 2.43 g/kg for hypertensive animals and those put on a 4-week diet to reverse hypertension, respectively). A more aggressive therapy for lowering blood pressure, the administration of chlorothiazide in addition to a low sodium diet, resulted in a more rapid normalization of blood pressure (2–3 weeks with chlorothiazide and diet vs 4–6 weeks with dietary sodium restriction alone). Nevertheless, the percentage of cells with tetraploid nuclei still remained elevated.

### Table 1. Effect of DOC-Salt Hypertension and Its Reversal on Blood Pressure and Ploidy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood pressure (mm Hg)</th>
<th>Tetraploid nuclei (% of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>DOC and salt (4 wk)</td>
<td>122.1 ± 2.8</td>
<td>207.4 ± 6.0</td>
</tr>
<tr>
<td>DOC and salt (4 wk) + low-Na diet (8 wk)</td>
<td>120.0 ± 1.1</td>
<td>118.8 ± 1.1</td>
</tr>
<tr>
<td>DOC and salt (4 wk) + low-Na diet (13 wk)</td>
<td>100.0 ± 1.8</td>
<td>116.6 ± 6.3</td>
</tr>
<tr>
<td>DOC and salt (4 wk) + low-Na diet and chlorothiazide (8 wk)</td>
<td>120.2 ± 5.0</td>
<td>116.3 ± 2.0</td>
</tr>
</tbody>
</table>

All values represent means ± SE for groups containing five to nine rats. Significant differences in ploidy between the control and treated groups were determined by a two-way analysis of variance.
Discussion

These studies show that DOC-salt hypertension leads to a rapid and marked increase in the number of smooth muscle cells with tetraploid nuclei in aortic tissue. Previous studies have documented an increase in aortic polyploid cells as a response to hypertension in SHR and two-kidney, one clip Goldblatt rats.1,3 The DOC-salt model differs from the SHR and Goldblatt models in that the maximum percentage of cells with tetraploid nuclei occurred after 4 weeks of treatment and remained unchanged as hypertension persisted. In contrast, polyploidy increased less rapidly, over a 6-month period, in the other animal models of hypertension, and this less rapid rise appeared to be paralleled by a more gradual rise in blood pressure.

Whether intact cells or isolated nuclei obtained from these cells were analyzed by flow cytometry to determine nuclear ploidy, the results were similar. For technical reasons, it is advantageous to analyze nuclei rather than whole cells, because the resolution is better and such problems as clumping of noncellular material introduced into the flow cytometer are minimized. The close agreement between the procedures argues against large numbers of binucleated cells in hypertensive vessels from DOC-salt-treated rats. Recently, Owens3 employed morphometric techniques to show that binucleated cells were present in aortic tissue from SHR but were far less numerous than tetraploid cells.

By measuring forward light scatter and analyzing the data by computer, we were able to determine that the average smooth muscle cell with a tetraploid nucleus was between 1.6- and 1.7-fold larger than the average cell with a diploid nucleus. The values are comparable to those reported by Owens and Schwartz,2 who used the relatively time-consuming method of scanning microdensitometry to quantitate the mass. The values are also close to the 1.59-fold increase in the volume of cells with tetraploid nuclei calculated on the assumption that the volumes of the nucleus and cytoplasm increase proportionally.

Reversal of DOC-salt hypertension was accompanied by a lowering of the heart/body weight ratio. Although treatment prevented further increases in the accumulation of cells with tetraploid nuclei, it did not result in a reversal of the ploidy changes observed, regardless of whether dietary sodium restriction and cessation of DOC treatment were used alone or in combination with a diuretic. This suggests that the blood pressure reduction itself was responsible for stabilization of the ploidy changes in the treated animals. Our findings confirm those of Owens,3 who found that treating hypertension in SHR with chlorothiazide, reserpine, and hydralazine prevented further increases in the percentage of polyploid cells but did not reverse the increases that had already developed.

It is not surprising that restoration of normal blood pressure did not result in a reversal of the ploidy changes observed. Recent studies have shown that tetraploid arterial smooth muscle cells, when cultured, remained tetraploid and did not revert to cells with diploid nuclei.4

The irreversibility of the hypertension-induced change in ploidy is also of interest in the context of our previous study2 showing that certain enzymatic changes in aortic tissue induced by hypertension also were not completely reversed either by drug treatment in the SHR model or dietary manipulation in the DOC-salt model. Further studies can utilize the cell-sorting capability of the flow cytometer to isolate the different cell populations and determine how hypertension affects the chemical and enzymatic characteristics of each cell type.

Acknowledgments

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References

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