An Increased Pool of Secretory Hormones and Peptides in Adrenal Medulla of Stroke-Prone Spontaneously Hypertensive Rats

Maria Schober, Peter R.C. Howe, Günther Sperk, Reiner Fischer-Colbrie, and Hans Winkler

Secretory components of the adrenal medulla were compared in normotensive Wistar-Kyoto (WKY) rats and in stroke-prone spontaneously hypertensive rats (SHRSP) at both 4 and 12 months of age. Noradrenaline, adrenaline, dopamine, neuropeptide Y, and chromogranins A and B were significantly higher in adrenal glands of SHRSP than those of WKY rats at 4 months. At 12 months, the levels of these components in SHRSP had increased even more (about 200% in WKY rats). There was no change in the relative composition of the adrenal "secretory cocktail." Neither the chromogranin A/chromogranin B ratio nor their apparent proteolytic processing in chromaffin granules differed between SHRSP or WKY rats. The lack of a significant change in membrane-bound cytochrome b₅₅₆, and the small increase in dopamine β-hydroxylase suggest that the higher levels of secretory components in SHRSP are not simply caused by an increase in the number of chromaffin granules, but possibly by a selective increase in the secretory content of these organelles providing a larger package for quantal release by exocytosis. This may be relevant for the elevation of blood pressure in this strain. The immunological methods described in this paper allow for the first time a determination of the secretory quantal levels in catecholamine storage. This should be useful for further studies in hypertensive models. (Hypertension 1989;13:469-474)

In a previous study, it was established that the plasma level of adrenaline is significantly elevated in stroke-prone spontaneously hypertensive rats (SHRSP) when compared with that in normotensive Wistar-Kyoto (WKY) rats. This was shown for adult rats, but also applies to rats 1 month of age (P.R.C. Howe, unpublished observation). Furthermore, drug treatment leading to the stimulation of the sympathetic nervous system induced a far greater elevation of plasma adrenaline in SHRSP than in WKY rats. Adrenaline is secreted from the adrenal medulla by a process of exocytosis that leads to the release of the total soluble content of chromaffin granules.

The best characterized chromaffin granules are those of bovine adrenal medulla, which contain a complex "secretory cocktail" that includes catecholamines, enkephalins, neuropeptide Y, and the so-called chromogranins, which are the major protein components. The chromogranins consist of two families, which have been named chromogranin A and B (for nomenclature, see Reference 7). Recent studies have indicated that the composition of this secretory cocktail can be modified by nervous and humoral influences (see Reference 8). Thus in rat adrenal glands, nervous activity leads to a significant increase of enkephalins and neuropeptides and their respective messenger RNA (mRNA), whereas chromogranin A is not changed under these conditions. On the other hand, levels of chromogranin A are influenced by hormones because its level and that of its mRNA strongly depend on the presence of glucocorticoids.

It appeared of interest to determine the levels of some of these components in the adrenal glands of SHRSP. We attempted to answer the following questions: 1) Do adrenal glands of SHRSP contain higher amounts of secretory components when compared with those of WKY rats? 2) Is there any change in the relative composition of the secretory cocktail? and 3) Is there a change in the number, the size, or the quantal content of chromaffin granules?
### Materials and Methods

#### Preparation of Tissue Homogenates

Male WKY rats and SHRSP aged 4 months and 12 months were obtained from the CSIRO breeding colony (Adelaide, Australia). The establishment of hypertension in SHRSP at both ages was confirmed by tail-cuff measurement of blood pressure. The systolic arterial blood pressure of 4-month-old rats was 119 ± 1 (n = 8) for WKY rats and 187 ± 2 mm Hg (n = 8) for SHRSP and at 12 months 130 ± 14 (n = 11) for WKY rats and 228 ± 10 mm Hg (n = 14) for SHRSP. Data on the development and the degree of high blood pressure have already been published. Adrenal glands were removed under nembutal anesthesia (sodium pentobarbitone, Ciba-Geigy, Basel, Switzerland, 40 mg/kg i.p.), which reduces adrenal secretion from the adrenal glands. The adrenal glands were snap frozen in liquid nitrogen, stored at −80°C for up to 1 month and then freeze-dried. The freeze-dried adrenal glands (a randomly chosen left or right gland for each animal) were homogenized in 400 µl of buffer (5 mM Tris/succinate, pH 5.9) by ultrasonication for 4 seconds. Antisera against rat chromogranin A (rabbit 868) and B (rabbit 69) were raised as described previously and used at a dilution of 1:200. It was established by extensive immunoblotting (see also Figure 2) that there is no cross-reaction between the antisera against chromogranin A and B. As a blocking reagent, 2% lipid-free instant milk plus 2% normal goat serum was used. Parameters to be compared (WKY rats vs. SHRSP) were always included in the same dot assay to avoid errors from interassay variation. Three to four concentrations of each sample were used. The regression coefficients were 0.93 ± 0.031 (mean ± SD, n = 30) for A and 0.953 ± 0.056 (n = 30) for B, which indicated a low intra-assay variability. The sample sizes used for dotting ranged from 0.06 to 0.9 µl of the original tissue homogenate.

Cytocrome b₅₆ and dopamine β-hydroxylase were determined by quantitative one-dimensional immunoblotting with specific antisera against bovine cytochrome b₅₆ (provided by D.K. Apps, Edinburgh, Scotland) and rat dopamine β-hydroxylase (gift from P. Fleming, Washington, DC). For these experiments, aliquots (up to 40 µl) of the ultrasonicated tissue homogenates (see section on preparation) were separated by one-dimensional polyacrylamide gel electrophoresis followed by immunoblotting by the method of Burnette with some minor modifications. Proteins

### Analytical Assays

Chromogranins A and B were determined by a dot immunobinding assay. Aliquots (25 µl) of the tissue homogenates were dissolved in 107 µl sample buffer plus sodium dodecyl sulfate (1% final concentration). After boiling for 5 minutes, Triton X-100 was added to a final concentration of 7% followed by ultrasonication for 4 seconds. Antiserum against rat chromogranin A (rabbit 868) and B (rabbit 69) were raised as described previously and used at a dilution of 1:200. It was established by extensive immunoblotting (see also Figure 2) that there is no cross-reaction between the antisera against chromogranin A and B. As a blocking reagent, 2% lipid-free instant milk plus 2% normal goat serum was used. Parameters to be compared (WKY rats vs. SHRSP) were always included in the same dot assay to avoid errors from interassay variation. Three to four concentrations of each sample were used. The regression coefficients were 0.93 ± 0.031 (mean ± SD, n = 30) for A and 0.953 ± 0.056 (n = 30) for B, which indicated a low intra-assay variability. The sample sizes used for dotting ranged from 0.06 to 0.9 µl of the original tissue homogenate.

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### Table 1. Content of Various Constituents in Adrenal Glands of Wistar-Kyoto Rats and Stroke-Prone Spontaneously Hypertensive Rats

<table>
<thead>
<tr>
<th>Components</th>
<th>WKY 4 mo</th>
<th>SHRSP</th>
<th>WKY 12 mo</th>
<th>SHRSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA (µg)</td>
<td>2.0±0.1</td>
<td>2.5±0.1*</td>
<td>1.8±0.2</td>
<td>4.9±0.4†</td>
</tr>
<tr>
<td>A (µg)</td>
<td>100±5</td>
<td>125±10</td>
<td>100±9</td>
<td>265±33</td>
</tr>
<tr>
<td>DA (µg)</td>
<td>16.2±0.6</td>
<td>20.1±0.9*</td>
<td>22.9±1.8</td>
<td>51.0±2.0†</td>
</tr>
<tr>
<td>NPY (pmol)</td>
<td>0±0.01</td>
<td>0.14±0.014</td>
<td>0.09±0.01</td>
<td>0.269±0.01†</td>
</tr>
<tr>
<td>Ch A (%)</td>
<td>100±8</td>
<td>133±13</td>
<td>100±10</td>
<td>302±34</td>
</tr>
<tr>
<td>Ch B (%)</td>
<td>100±5</td>
<td>129±10</td>
<td>100±10</td>
<td>265±33</td>
</tr>
<tr>
<td>DBH (%)</td>
<td>100±4</td>
<td>133±7†</td>
<td>100±4</td>
<td>198±101</td>
</tr>
<tr>
<td>Cyt (%)</td>
<td>100±5</td>
<td>131±14‡</td>
<td>100±9</td>
<td>220±21†</td>
</tr>
</tbody>
</table>

The results for norepinephrine (NA), adrenaline (A), and dopamine (DA) are expressed in micrograms per adrenal gland; those for neuropeptide Y (NPY) in picomoles per adrenal gland. For chromogranin A (Ch A), chromogranin B (Ch B), dopamine β-hydroxylase (DBH), and cytochrome b₅₆ (Cyt) absolute values could not be determined by the immunoblot procedure; therefore results are presented in percentages that give the differences in the levels between stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto (WKY) rats. Values are mean±SEM (n = 5–7) Standard error of the mean of percentages were calculated according to McLean and Welch. The significance of statistical difference by t test between WKY rats and SHRSP is given (*p<0.01, †p<0.001, p<0.05). n.d., not determined.

To compare several parameters with each other a one-way analysis of variance was performed for 12-month-old rats. The percentage levels in SHRSP of NPY, Ch A, and Ch B did not differ from each other, but each of them differed significantly from DBH and from Cyt, respectively. DBH did not differ significantly from Cyt.
were transferred to a nitrocellulose sheet via a transblot cell (Bio-Rad, Vienna, Austria) at constant 35 V for 18 hours. After transfer the replicas were incubated in a solution that contained 0.9% sodium chloride, 10 mM Tris-HCl, pH 7.2%, 2% liquid-free instant milk, and 2% goat serum at 37° C for 1 hour followed by incubation with antisera (diluted 200 x) for 3 hours at room temperature. After several washes, replicas were treated with iodine-125-labeled protein A (4 μCi/50 ml) for 1 hour at room temperature. The nitrocellulose sheet was washed to remove unbound [123I]protein A, blotted dry, and exposed to x-ray film at -70° C with intensifying screens for 1–2 days. The developed films were used to mark the antigen spots on the replicas. The relevant regions (and blanks of the same size) were then excised and counted for radioactivity in a gamma-spectrophotometer. The linearity of the reaction was tested for each sample with at least two concentrations. In general, the immune reaction was found to be linear up to 5,000 rpm.

For extraction of neuropeptide Y, 20 /il of the tissue homogenate was added to 1.0 ml of 2 M acetic acid, sonicated, centrifuged, and lyophilized. Neuropeptide Y (NPY) was determined by radioimmunoassay using a rabbit antiserum raised toward synthetic NPY (Cambridge Research Biochemicals, Cambridge, England) as described.16 In adrenal medulla, as demonstrated by high-pressure liquid chromatography, there is only one NPY-immunoreactive peak corresponding to synthetic NPY. Catecholamines were determined by reversed-phase high-performance liquid chromatography with electrochemical detection. 17

**Results**

The levels of the various constituents in adrenal medullae of WKY rats or SHRSP are shown in Table 1. At 4 months of age there is a significant difference between WKY rats and SHRSP in all parameters (i.e., noradrenaline, adrenaline, NPY, and chromogranin A and B); the increase is about 30%. At 12 months of age this difference rose by at least 98%. Figure 1 presents the results relative to the values for WKY rats at 4 months of age. In WKY rats, the levels of the various components (with the exception of noradrenaline and dopamine) increase during the aging of the rats. SHRSP start at an already high level at 4 months of age. There is a steeper increase when compared with WKY rats for all components. Dopamine and noradrenaline exhibit the lowest rises.

Chromogranin A and B increased to a similar degree. Therefore, there was no significant change in the ratio of chromogranin A to B when comparing WKY rats (ratio, 1.15±0.08, n=6, mean±SEM at 4 months and 1.37±0.22, n=5 at 12 months) with SHRSP (ratio, 1.34±0.14, n=7 at 4 months and 1.1±0.13, n=5 at 12 months). These peptides were determined quantitatively by an immunoblot procedure. An example of an immunoblot is shown in Figure 2. It is obvious that increased amounts of chromogranin A and B are present in SHRSP;

![FIGURE 1. Levels of various components in adrenal glands of Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP) at 4 and 12 months. Levels of WKY rats at 4 months were taken as 100%; levels of WKY rats at 12 months and of SHRSP at 4 and 12 months are expressed relative to this value. NPY, neuropeptide Y; Ch A, chromogranin A; A, adrenaline; Ch B, chromogranin B; DA, dopamine; NA, noradrenaline.](http://hyper.ahajournals.org/)

however, the patterns given by the proprotein plus the endogenous breakdown products are apparently unchanged. In addition to these secretory components, we also determined the levels of cytochrome b566, a typical membrane constituent of chromaffin granules.6 In 12-month-old SHRSP (see Table 1) the level of this protein was slightly higher in comparison with WKY rats; however, this difference was not significant. Finally, dopamine β-hydroxylase, a component present both in the content and the membranes of chromaffin granules,5 was determined. The level of this protein was significantly higher (by 53%) in SHRSP (see Table 1).

**Discussion**

This study has established that adrenal glands of SHRSP rats contain significantly higher levels of various secretory components when compared with those of WKY rats. In a previous study,1 it was suggested that the adrenal medulla was a source of elevated plasma levels of noradrenaline and adrenaline under normal and drug-induced conditions in SHRSP. In agreement with those findings, we found...
high levels of both catecholamines in adrenal glands of SHRSP in the present study. However, noradrenaline (and dopamine) in both WKY rats and SHRSP increased less with age than the other secretory components. Since adrenaline and noradrenaline are stored in separate cells, this may indicate a difference in the development of these two cell populations. The NPY levels in SHRSP adrenal glands were elevated to the same degree as adrenaline. These two secretory constituents are apparently co-localized in the same cells. In the previous study on plasma levels, NPY in plasma differed from the catecholamines. Levels were only slightly raised and drug-induced increases were much less significant than those for the catecholamines. In this study the authors suggested that sampling for NPY in the plasma at a certain point may have missed the full NPY response. This might explain the obvious lack of increase in NPY levels in plasma despite high levels in the adrenal glands. However, most of the NPY may be derived from the sympathetic nerve, and thus release from the adrenal glands may not contribute significantly to a high background level coming from nervous activity. Our results in the present study show that adrenal glands contain increased levels not only of catecholamines and one neuropeptide, but also of the major secretory components of chromaffin granules (i.e., the chromogranins A and B).

We already discussed that increased nervous firing to the adrenal glands changes the composition of the secretory cocktail in these organs, which leads to a relative rise in enkephalins and NPY levels. However, in SHRSP there was no evidence that NPY was preferentially increased. This argues against an increase of nervous activity in these rats. However, it cannot be excluded that a prolonged elevation of nervous activity induces changes that are different from those after a more limited stimulation such as those used under previous experimental conditions. There was also no indication that the relative amounts of other components were changed (e.g., from a different activity of the adrenal cortex one might have expected a change in the relative levels of adrenaline or chromogranin A, both of which depend on glucocorticoids for their synthesis). Finally, there was no indication that the proteolytic processing within chromaffin granules was enhanced because the relative pattern of chromogranin A breakdown products was identical in WKY rats and SHRSP. It seems also unlikely that the processing of neuropeptides (e.g., neuropeptide Y) is different between normal and hypertensive rats.
Our results establish that in SHRSP the adrenal pool of secretory components is higher than in WKY rats. What is the cellular mechanism behind this? One might suggest that this is simply an indication of hypertrophy wherein the adrenal glands contain more or larger cells and therefore more chromaffin granules. The increase in weight in adrenal glands of SHRSP at 12 months apparently supports such a possibility. However, our data on cytochrome b$_{59}$ and dopamine $\beta$-hydroxylase indicate that the situation is more complex. The cytochrome is a major constituent of the membrane of these organelles, although it is absent from the secretory content. It therefore represents a good marker for the amount of granule membranes present. In SHRSP there was a slightly higher level of cytochrome; however, this difference was not significant and was much lower than that in the levels of the secretory components. This does not support the idea that merely an increase in the number of chromaffin granules can explain the elevation of the secretory components since in this case the cytochrome should have increased in parallel with the secretory components. Finally, levels of dopamine $\beta$-hydroxylase were determined. In rat chromaffin granules, 80% of the enzyme is membrane-bound, and the rest is found in the secretory content. The total enzyme activity was higher by 53%, which is more than the difference in cytochrome levels, but significantly less than that of the secretory components confined to the soluble content. Assuming that the soluble portion of dopamine $\beta$-hydroxylase goes up by a factor of about two, the observed difference is consistent with an elevation of the amounts of membranes by about 20% as shown by the cytochrome value.

Thus all these data are consistent but do not yet prove the concept that in SHRSP the number of chromaffin granules is increased slightly or the granules have become larger. If the content goes up by 100%, one can calculate that surface membranes containing the cytochrome b$_{59}$ (80% of the dopamine $\beta$-hydroxylase) increase only by 53%. However, since cytochrome b$_{59}$ was elevated only by 24%, neither an increase in number nor an enlargement of the granules can explain the marked rise in secretory components. Therefore, an additional mechanism, that the granules are filled with more secretory products that make the size of the secretory quantum larger, must be considered. Morphometric studies should now be undertaken to investigate the number of cells and chromaffin granules in the adrenal glands of SHRSP and to determine the size of the granules.

The present study has established immunological methods that, for the first time, allow an estimation of the secretory quantum involved in catecholamine release. Further studies on sympathetic nerves should use such methods to ascertain whether a change in secretory quantum amount could play a significant role in experimental and pathological cases of hypertension. Finally, a causal relation between high blood pressure and a change in secretory quantum levels in SHRSP could only be established by an appropriate genetic analysis.  

References


**KEY WORDS** • neuropeptide Y • catecholamines • adrenal gland • chromogranin A • chromogranin B • stroke-prone spontaneously hypertensive rats
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