Catecholamine Secretory Vesicles
Augmented Chromogranins and Amines in Secondary Hypertension

Marwan A. Takiyyuddin, Luca De Nicola, Francis B. Gabbai, Thai Q. Dinh, Brian Kennedy, Michael G. Ziegler, Esther L. Sabban, Robert J. Parmer, and Daniel T. O'Connor

Chromogranins A and B are major soluble proteins in chromaffin granules. Their adrenomedullary content is increased in the spontaneously (genetic) hypertensive rat. Is augmented catecholamine vesicular storage of the chromogranins a specific feature of genetic hypertension? To explore this question, we measured chromogranin A immunoreactivity, using a novel, synthetic peptide radioimmunoassay, in rat adrenal medullas 4–6 weeks after induction of the two-kidney, one clip Goldblatt model of renovascular hypertension and in unmanipulated control animals. We also measured messenger RNAs of chromogranins A and B and dopamine β-hydroxylase by Northern blot. Immunoreactive RNAs of chromogranin A was 3.3-fold higher (p<0.01) in clipped rat adrenals. Adrenal catecholamine concentrations and phenylethanolamine-N-methyltransferase activity were also higher in clipped rats. Adrenal dopamine β-hydroxylase activity (both membrane-bound and soluble forms) and corticosterone (glucocorticoid) concentration did not significantly differ between the groups. Adrenal medullary chromogranin A messenger RNA levels in clipped rats were 3.2-fold higher (p=0.029) than those in the control group, and chromogranin B messenger RNA levels were 4.6-fold higher (p=0.05). Dopamine β-hydroxylase messenger RNA levels were 2.9-fold higher (p=0.038). Thus, augmented synthesis and storage of adrenomedullary chromogranins A and B, catecholamines, and their biosynthetic enzymes appear to be characteristic of both acquired and genetic hypertension. (Hypertension 1993;21:674–679)

**KEY WORDS** • chromogranins • adrenal medulla • catecholamines • chromaffin granules • hypertension, Goldblatt

The chromogranins/secretogranins are a family of acidic, soluble proteins initially described in adrenomedullary chromaffin vesicles but subsequently found in the core of virtually all neuroendocrine secretory vesicles.1-6 The relative abundance of the chromogranins in chromaffin vesicles appears to be species dependent, with chromogranin A (CgA) and chromogranin B (CgB) being quantitatively major proteins in bovine and human vesicles.7 Schober et al8 recently reported an increase in the adrenal medullary content of chromogranins (including CgA and CgB) as well as catecholamines in the stroke-prone spontaneously (genetic) hypertensive rat compared with its normotensive Wistar-Kyoto control strain.

Is augmented catecholamine vesicular storage of the chromogranins a specific feature of genetic hypertension? To explore this question, we developed a novel, rapid, sensitive, and specific radioimmunoassay for rodent CgA, based on CgA synthetic peptides, and applied the assay to adrenal medullas from animals with experimental Goldblatt (two-kidney, one clip [2K1C]) renovascular hypertension. Furthermore, we assessed CgA and CgB messenger ribonucleic acid (mRNA) levels. Our results suggest that biosynthesis and storage of chromogranins are augmented in acquired as well as genetic hypertension.

**Methods**

**Animals**

Studies were performed in male Munich-Wistar rats (180–220 g body wt) obtained from Simonsen Laboratories, Gilroy, Calif. 2K1C Goldblatt model rats were prepared with rats under methohexital sodium anesthesia by placing a 0.2-mm slit-width silver clip on the right renal artery.9 Systolic blood pressure was measured weekly in awake clipped animals via the tail-cuff method with an electrosphygmomanometer (Narco BioSystems, Austin, Tex.) in a heated animal restraining cage. Clipped rats with systolic blood pressure >140 mm Hg were further studied (n=16). Normotensive, unmanipulated rats of the same age, gender, weight, and strain served as controls (n=10). Animals were maintained with ad...
libitum access to tap water and normal rat chow until the morning of the experiment.

Four to 6 weeks after renal artery clipping, rats were anesthetized with Inactin (10 mg per 100 g body wt i.p.). Mean arterial pressure was measured with a transducer (model P23db, Statham Instruments, Gould Division, Hato Rey, Puerto Rico) connected to a femoral artery catheter. Adrenals were surgically removed, frozen in liquid nitrogen, and stored at −70°C until processed. Animals were subsequently killed with use of sodium brevital (300 mg/kg i.p.).

Tissues and Proteins

Homogenates from rat adrenals and chromaffin vesicle lysates from human pheochromocytoma and normal bovine adrenal medulla were prepared as previously described. Bovine CgA was isolated from an adrenal chromaffin vesicle soluble lysate as previously described.

Peptides

Peptide hapten were synthesized by the solid-phase method and isolated by reversed-phase high performance liquid chromatography (HPLC) on a 10x250-mm semi-preparative C18 (octadecasyllic) column, using a 0–60% gradient of acetonitrile in 0.1% trifluoroacetic acid.

The peptides synthesized included bovine/human CgA N-terminus [bovine/human CgA(N-179)[Tyr17]]; LPVNSP-MNKGDTEVMKY] and rat CgA N-terminus [CgA(N-95)[Tyr9]]; LPVNSPMTY]. The structure of each peptide was verified by analysis of amino acid composition of 24-hour HCl hydrolysates. The terminal adventitious tyrosine residues enabled coupling of the hapten to a carrier protein (keyhole limpet hemocyanin; Calbiochem Corp., La Jolla, Calif.) by Tyr-Tyr cross-linking using bis-diazotized benzidine, as well as radioiodination.

The HPLC-purified N-terminal 17-mer (LPVNSP-MNKGDTEVMKY) was iodinated with 125I-Na by the chloramine-T method.

Catecholamine concentrations and phenylethanolamine-N-methyltransferase (PNMT) activity were determined radioenzymatically. DBH activity, in membrane-bound and soluble forms in chromaffin granules, was determined spectrophotometrically. Membrane and soluble fractions of adrenal medulla were separated by centrifugation of adrenal homogenates in 10 mM sodium phosphate, pH 6.5, at 13,000g for 1 hour at 4°C. DBH results are expressed as international units, where 1 unit represents conversion of 1 μmol of tyramine substrate to octopamine product per minute at pH 5.0 and 37°C.

Protein concentration in adrenal homogenates was determined by the Coomassie blue dye binding method, and corticosterone (the rodent glucocorticoid) was determined by radioimmunoassay (ICN Biomedicals, Inc., Costa Mesa, Calif.).

Statistical Analysis

Results are expressed as mean±SEM. Data were analyzed by analysis of variance. A value of p<0.05 was considered statistically significant.
Antibody

0.001 0.01 0.1 1 10
Chromaffin tissue fraction, µl

Chromogranin A N-terminal 17-mer
Chromogranin A, bovine whole molecule
Human pheochromocytoma chromaffin vesicle lysate (2200-7)
Rat adrenal homogenate
Confluence of symbols

Chromogranin A, moles/tube

FIGURE 1. Plot shows standard curve of chromogranin A (CgA) radioimmunoassay. Curves represent parallel displacement of radiolabeled CgA N-terminal synthetic peptide (CgA<sub>N-term</sub><sup>125I</sup>-[Tyr<sup>17</sup>]) from specific antibody by unlabeled peptide standard or tissue homogenates. The assay standard curve is plotted as log<sub>10</sub> of standard antigen concentration (horizontal axis) versus B/B<sub>o</sub> (vertical axis), where B is counts per minute precipitated in any given assay tube, and B<sub>o</sub> is counts per minute precipitated without addition of unlabeled standard antigen.

at antiserum titers binding 30–50% of the labeled peptide, constituted the radioimmunoassay standard curve (Figure 1). The lower limit of detection in this radioimmunoassay (defined as the unlabeled antigen dose where B=80% of B<sub>o</sub>) was 2×10<sup>-12</sup> mol of CgA per assay tube. The assay had intra-assay and interassay coefficients of variation of 4.8% (n=12) and 8.4% (n=24), respectively.

Renal Artery Clipping

Four to 6 weeks after right renal artery clipping, mean arterial pressure ranged from 157 to 200 mm Hg (mean, 177±3 mm Hg) on the day of experiment in the animals with the clipped kidney and from 105 to 120 mmHg (mean, 111±3 mm Hg) in the control group (p<0.001).

Immunoreactive adrenal CgA was 3.3-fold higher (p=0.003) in rats with clipped kidneys (n=11) than in control rats (n=5, Table 1). Adrenal PNMT activity also increased approximately 45% (p<0.05) after renal artery clipping, whereas the increments in adrenal epi-

TABLE 1. Levels of Chromogranin A, Catecholamines, Catecholamine Bio
synthetic Enzymes, and Corticosterone in Rat Adrenals

<table>
<thead>
<tr>
<th>Adrenal values</th>
<th>2KIC (n=11)</th>
<th>Control (n=5)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CgA (pmol/mg protein)</td>
<td>402±50</td>
<td>122±35</td>
<td>0.003</td>
</tr>
<tr>
<td>E (µg/mg protein)</td>
<td>5.3±0.4</td>
<td>3.7±0.6</td>
<td>0.052</td>
</tr>
<tr>
<td>NE (ng/mg protein)</td>
<td>887±69</td>
<td>636±102</td>
<td>0.062</td>
</tr>
<tr>
<td>PNMT (pmol/hr/mg protein)</td>
<td>761±57</td>
<td>525±92</td>
<td>0.042</td>
</tr>
<tr>
<td>DBH (mIU/mg protein)</td>
<td>Membrane-bound</td>
<td>11.8±1.9</td>
<td>9.18±1.8</td>
</tr>
<tr>
<td>Soluble</td>
<td>2.51±0.5</td>
<td>2.54±0.4</td>
<td>0.94</td>
</tr>
<tr>
<td>Corticosterone (µg/mg protein)</td>
<td>23.41±3.77</td>
<td>23.18±4.37</td>
<td>0.97</td>
</tr>
</tbody>
</table>

2KIC, two-kidney, one clip Goldblatt hypertensive rats; CgA, chromogranin A; E, epinephrine; NE, norepinephrine; PNMT, phenylethanolamine-N-methyltransferase; DBH, dopamine β-hydroxylase. Values are mean±SEM. Animals were studied 4–6 weeks after renal artery clipping.
2 K 1 C mRNA analysis

![Northern blot analysis of adrenal medullary messenger RNAs (mRNA)](image)

FIGURE 2. Northern blot analysis of adrenal medullary messenger RNAs (mRNA). mRNA (1.1 µg) was loaded into each lane. Positions of 18S and 28S forms of ribosomal RNA are indicated on the left. 2K1C, two-kidney, one clip (Goldblatt) hypertension; Control, unmanipulated rats; CgA, chromogranin A; CgB, chromogranin B; DBH, dopamine β-hydroxylase.

present study, we asked whether augmented adrenomedullary chromogranin and catecholamine storage is a specific feature of genetic hypertension.

The cDNA-deduced primary structures of the chromogranins are now known.29-30 The availability of the full-length primary structure of CgA allowed us to formulate a rodent CgA radioimmunoassay based on synthetic peptides and subsequently to probe alterations in CgA storage and synthesis in an experimental model of secondary (2K1C) hypertension.

The radioimmunoassay recognized authentic CgA, as judged from parallel displacement of synthetic tracer (CgAα16)-125I-[Tyr17]) by both intact CgA and the CgA N-terminal peptide (Figure 1). Both human and rodent CgA were recognized in parallel by this radioimmunoassay (Figure 1).

Renal artery clipping increased adrenal mRNAs encoding CgA, CgB, and DBH (Figure 2), as well as the adrenal content of CgA and catecholamines and the activity of the epinephrine biosynthetic enzyme PNMT (Table 1).

What factors in 2K1C hypertension mediated increased adrenal synthesis and storage of chromogranins and catecholamines? CgA expression is regulated by glucocorticoids,31 but corticosterone (rat glucocorticoid) levels were unaltered in 2K1C rats (Table 1), precluding a role for endogenous glucocorticoids in upregulation of CgA and CgB biosynthesis here.

Increased efferent sympathetic nervous activity is a reproducible finding in 2K1C hypertension.32,33 The activity of adrenal and vascular tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, is increased in 2K1C hypertension, suggesting increased efferent sympathetic neuronal traffic to the adrenal medulla in particular.33 Because tyrosine hydroxylase is subject to transsynaptic induction by efferent stimulation of chromaffin cells,34,35 the increase in adrenal catecholamine storage in 2K1C hypertension may reflect an increase in splanchnic tone.

Does enhanced splanchnic nerve traffic to the adrenal medulla upregulate chromogranin biosynthesis as well? Preganglionic sympathetic axons stimulate chromaffin cells by release of the ganglionic neurotransmitter acetylcholine, which interacts with nicotinic cholinergic receptors on the chromaffin cell surface to provoke exocytotic secretion.36 In vitro, nicotinic cholinergic stimulation of chromaffin cells increases incorporation of 35S-methionine into newly synthesized CgA; that is, CgA biosynthesis is augmented.37 Thus, the increased efferent splanchnic sympathetic nerve traffic in 2K1C hypertension may trigger increased biosynthesis of the chromogranins as well as of the catecholamines.

In vivo, CgA biosynthesis has been studied after reflex splanchnic stimulation by hypoglycemia or reserpine.31,38,39 The effects of such stimulation on CgA biosynthesis have been variable.31,38,39

Adrenal medullary PNMT40-42 and DBH41-43 activities are also augmented by neural stimuli.34,35,40-43 The rise in adrenal DBH with repeated stress was recently shown to be mediated by elevated mRNA levels.44 PNMT activity was increased by renal artery clipping (Table 1), whereas membrane-bound DBH activity was somewhat elevated but not significantly. However, although catecholamine biosynthetic enzymes such as tyrosine hydroxylase, DBH, and PNMT all increase after neural (transsynaptic) stimuli, their responses vary in magnitude.41-43 The discrepancy between the 2.9-fold rise in DBH mRNA levels and the approximately 30% (but not significant) elevation of DBH activity is somewhat puzzling, especially because the amount of immunoreactive CgA corresponds to the elevation in its mRNA levels. It is possible that the mRNA does not correspond to increased DBH activity levels,
because of corresponding changes in the half-life of DBH in the rats with the clipped kidney. Alternatively, DBH activity assays, which are subject to endogenous inhibitors, may not reflect the in vivo activity. It should be noted, however, that although there were no significant changes in DBH activity in the adrenals of the hypertensive animals, because of the large standard error, the elevation in membrane-bound DBH activity (29%) is not that different in magnitude from the 40–45% increase in adrenal catecholamine levels and in PNMT activity or from the reported rise of adrenal tyrosine hydroxylase activity (49%) in 2K1C hypertensin.33

The increment in adrenomedullary catecholamines after renal artery clipping was statistically marginal (p = 0.052 to 0.062, Table 1). By contrast, Schober et al reported more significant (p<0.01) increments in adrenal catecholamines in the stroke-prone spontaneously hypertensive rat. However, adrenal catecholamine contents in the spontaneously hypertensive rat are controversial, with some authors reporting unchanged or even diminished values.45,46

In conclusion, the adrenomedullary content of chromogranins and catecholamines is augmented in experimental Goldblatt (2K1C) hypertensin. The increment in chromogranin storage appears to be the result of upregulated chromogranin synthesis. Thus, increased adrenomedullary storage and synthesis of chromogranins appear to be characteristic of acquired as well as of genetic hypertensin.

Acknowledgment

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References

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