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.1,2 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 electrophysmomanometer (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.10-11 Bovine CgA was isolated from an adrenal chromaffin vesicle soluble lysate as previously described.10-11

**Peptides**

Peptide haptons were synthesized by the solid-phase method12 and isolated by reversed-phase high performance liquid chromatography (HPLC) on a 10x250-mm semipreparative C18 (octadecasyl) 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(1-17)[Tyr17]; LPVNSPMNKG-DTEVMKTY] and rat CgA N-terminus [CgA(1-17)[Tyr17]; LPVNSP-MNKG-DTEVMKTY]. The structure of each peptide was verified by analysis of amino acid composition of 24-hour HCl hydrolysates.4 The terminal adventitious tyrosine residues enabled coupling of the haptons to a carrier protein (keyhole limpet hemocyanin; Calbiochem Corp., La Jolla, Calif.) by Tyr-Tyr cross-linking using bis-diazobenzidine,13 as well as radioiodination.14

The HPLC-purified N-terminal 17-mer (LPVNSP-MNKG-DTEVMKTY) was iodinated with 125I-Na by the chloramine T method.14 The labeled peptide (2 μg) was isolated from unincorporated radioiodide by adsorption to and elution from an octadecasyl cartridge (Sep-Pak C18, Waters Chromatography Division, Milford, Mass.). The radiolabeled peptide (mean specific activity, 2.2×106 cpm/μg [n = 3; triplications]) was stored diluted in buffer solution at −70°C for 4–6 weeks with little loss of immunoreactivity.

**Antisera**

Rabbit polyclonal anti-peptide antisera were developed to the carrier-conjugated peptide haptons as previously described.16,17

**Chromogranin A Immunoprecipitation and Radioimmunoassay**

Immunoprecipitation and radioimmunoassay of the radiolabeled N-terminal CgA peptide, CgA(1-17)[125I-Tyr17], by rabbit polyclonal anti-peptide antisera was accomplished as previously outlined for immunoprecipitation of intact 125I-labeled CgA.18

The optimal (working) titer of first antibody for radioimmunoassay was that which immunoprecipitated 30–40% of 10,000 cpm of the 125I-labeled antigen. For assay antibody No. 625-4, the final titer was 1:180 (vol/vol). Immunoreactive CgA was determined from parallel displacement of tracer by unlabeled standard and unknown sample (tissue homogenate) from the antibody in the radioimmunoassay. The assay standard curve was plotted as log10 of the unlabeled standard antigen concentration versus B/B0, where B0 is the counts per minute precipitated without addition of unlabeled standard antigen, and B is the counts per minute precipitated in any given assay tube.

**Northern Blot Analysis of mRNAs**

Total adrenal RNA was extracted from frozen tissue by the guanidinium thiocyanate method.16 mRNAs were isolated on an oligo-dT cellulose column and subsequently analyzed (1.1 μg per lane) by hybridization with the following random primer-labeled complementary DNA (cDNA) probes, as previously described:1) a rat CgA cDNA 1.6-kbp fragment spanning the entire open reading frame;2) a 2.3-kbp full-length rat CgB cDNA,3 and 3) a 1.286-bp rat dopamine β-hydroxylase (DBH) cDNA.23

Between probes, the filters were stripped by treatment in 80% formamide/20% water at 55°C for 15 minutes twice. They were then soaked in 0.1× saline–sodium phosphate–EDTA (SSPE) buffer at room temperature for 20 minutes and rinsed briefly in 5× SSPE before reprobing.24 Northern blot autoradiographic bands were quantified by scanning the autoradiograms with a transmission densitometer (LKB, Bromma, Sweden).**Other Assays**

Catecholamine concentrations and phenylethanolamine-N-methyltransferase (PNMT) activity were determined radioenzymatically.25 DBH activity, in membrane-bound and soluble forms in chromaffin granules, was determined spectrophotometrically.26 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,27 and corticosterone (the rodent glucocorticoid) was determined by radioimmunoassay (ICN Biomedicals, Inc., Costa Mesa, Calif.).28

**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.

**Results**

**Development of an Immunoassay for Rodent Chromogranin A**

With repeated immunization, carrier-conjugated N-terminal synthetic CgA peptides provoked antisera whose titers for 50% binding of 125I-labeled intact CgA were in the range of 1:30 to 1:1,000 (vol/vol, data not shown).

Displacement of CgA(1-17)[125I-Tyr17] from anti-N-terminal peptide antisera by unlabeled CgA(1-17)[Tyr17],
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 \(B/B_0=0.8\)) was \(2 \times 10^{-12}\) 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 mm Hg (mean, 111±3 mm Hg) in the control group (\(n=5\) each). The difference was statistically significant (\(p<0.05\)), in the clipped animals (\(n=5\)) than in controls (\(n=5\)).

Discussion

The chromogranins/secretogranins are a family of acidic, soluble proteins found in the core of chromaffin vesicles. Augmented adrenomedullary storage of the chromogranins has been demonstrated in genetic hypertension (the stroke-prone spontaneously hypertensive rat). However, the factor or factors underlying increased chromogranin storage have not been elucidated. In the

<table>
<thead>
<tr>
<th>Chromogranin A, moles/tube</th>
<th>Antibody %625-4</th>
</tr>
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<tbody>
<tr>
<td>Chromogranin A N-terminal 17-mer</td>
<td>[Tyr17]</td>
</tr>
<tr>
<td>Chromogranin A, bovine whole molecule</td>
<td></td>
</tr>
<tr>
<td>Human pheochromocytoma chromaffin vesicle lysate (2200-7)</td>
<td></td>
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<tr>
<td>Rat adrenal homogenate</td>
<td></td>
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<td>Confluence of symbols</td>
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FIGURE 1. Plot shows standard curve of chromogranin A (CgA) radioimmunoassay. Curves represent parallel displacement of radiolabeled CgA N-terminal synthetic peptide (CgA N-terminal{[Tyr17]}{[Tyr17]}) from specific antibody by unlabeled peptide standard or tissue homogenates. The assay standard curve is plotted as log \(B/B_0\) of standard antigen concentration (horizontal axis) versus \(B/B_0\) (vertical axis), where \(B\) is counts per minute precipitated in any given assay tube, and \(B_0\) is counts per minute precipitated without addition of unlabeled standard antigen.

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

<table>
<thead>
<tr>
<th>Adrenal values</th>
<th>2KIC</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CgA (pmol/mg protein)</td>
<td>402±50</td>
<td>122±35</td>
</tr>
<tr>
<td>E ((\mu g/mg) protein)</td>
<td>5.3±0.4</td>
<td>3.7±0.6</td>
</tr>
<tr>
<td>NE (ng/mg protein)</td>
<td>887±69</td>
<td>636±102</td>
</tr>
<tr>
<td>PNMT (pmol/hr/mg protein)</td>
<td>761±57</td>
<td>525±92</td>
</tr>
<tr>
<td>DBH (mIU/mg protein)</td>
<td>11.8±1.9</td>
<td>9.18±1.8</td>
</tr>
<tr>
<td>Membrane-bound</td>
<td>2.5±0.5</td>
<td>2.54±0.4</td>
</tr>
<tr>
<td>Soluble</td>
<td>23.4±3.77</td>
<td>23.18±4.37</td>
</tr>
<tr>
<td>Corticosterone ((\mu g/mg) protein)</td>
<td>20.4±3.4</td>
<td>23.18±4.37</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 \(\beta\)-hydroxylase. Values are mean±SEM. Animals were studied 4–6 weeks after renal artery clipping.
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 (CgA[1-16])-[125]Tyr1 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 splanchic tone.

Does enhanced splanchic 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 splanchic sympathetic nerve traffic in 2K1 hypertension may trigger increased biosynthesis of the chromogranins as well as of the catecholamines.

In vivo, CgA biosynthesis has been studied after reflex splanchic stimulation by hypoglycemia or reserpine.31,38,39 The effects of such stimulation on CgA biosynthesis have been variable.31,38,39 Adrenal medullary PNMT and DBH activities are also augmented by neural stimuli.41-43 The rise in adrenal DBH with repeated stress was recently shown to be mediated by elevated mRNA levels.41,42 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 hypertension.33

The increment in adrenal catecholamines after renal artery clipping was statistically marginal (p=0.052 to 0.062, Table I). By contrast, Schober et al8 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 (2KIC) hypertension. 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 hypertension.

Acknowledgment

CgA peptides were synthesized in the Center for Molecular Genetics, University of California, San Diego.

References

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