

Age-Related Increase of Calcitonin Gene-Related Peptide in Rat Thyroid and Circulation

SUNIL J. WIMALAWANSA

Biochemical Endocrinology Unit, Department of Chemical Pathology, Royal Postgraduate Medical School
Du Cane Road, London W12 ONN, UK

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WIMALAWANSA, S. J. *Age-related increase of calcitonin gene-related peptide in rat thyroid and circulation*. PEPTIDES 12(5) 1143-1147, 1991.—Elevated calcitonin levels in thyroid gland extracts and in plasma accompanied by C-cell hyperplasia are frequently found in old rats, in particular those raised in laboratory conditions. In parallel with calcitonin, we demonstrate here that the thyroidal content and plasma levels of immunoreactive calcitonin gene-related peptide (i-CGRP) significantly increase with age in rats ($p < 0.0001$). C18 Sep-Pak-extractable i-CGRP level in plasma was 35% of the total i-CGRP. Gel permeation chromatography and rp-HPLC studies revealed a number of immunoreactive molecular forms of CGRP and only 40-50% of the acid-extracted immunoreactivity was coeluted with the synthetic CGRP(1-37). The i-CGRP level measured in plasma was highly correlated with the thyroidal content of CGRP ($p < 0.001$) and also with the age of the rat ($p < 0.001$), suggesting an age-related increase of contribution of CGRP from thyroid gland into the circulation.

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| Calcitonin gene-related peptide (CGRP) | Plasma | Thyroid | Chromatography | Radioimmunoassay |
| Age related | | | | |

THE existence of calcitonin gene-related peptide (CGRP) was predicted (14) and later proven by its isolation and characterization from medullary thyroid carcinoma (MTC) (11). The first isolation and full characterization of CGRP from "normal" human tissue was reported recently (29). CGRP is widely distributed in rat tissues (14,24), and its presence has been shown in central and peripheral nervous tissues (19, 20, 24) and a variety of other tissues, including cardiovascular tissues (26) and thyroid (7, 16, 20), and in the C-cells colocalized with calcitonin (CT) (7). The role of CGRP in the circulation and the significance of its presence in the thyroid gland is yet to be determined.

CGRP is a potent vasodilator (3,5) and its distribution in perivascular nerves (14) and the abundance of its receptors in the cardiovascular system (18,26) imply that it may play a central part in the regulation of peripheral vascular tone. The presence of immunoreactive CGRP (i-CGRP) has been shown in rat (27,31) and in human plasma (6,22). The existence of multiple molecular weight (mol.wt.) forms of i-CGRP has been reported in human cerebrospinal fluid (25) and in plasma of rats treated with capsaicin (27). The present study demonstrates that existence of multiple mol.wt. forms of i-CGRP in normal rat plasma, and relative changes of i-CGRP in thyroid gland and plasma with ageing.

METHOD

Preparation of Plasma

Following an overnight fast, male Wistar rats were anesthetized with ether and exsanguinated via the dorsal aorta into cooled heparinized syringes. To avoid the effects of possible di-

urnal variation of circulating CGRP (23), all rats were bled between 10-11:00 a.m. Aprotinin (Trasylol 100 KIU/ml of blood) was added and the plasma separated. Samples of (500 μ l \times 2) plasma were kept for radioimmunoassay (RIA) and a further 2-4 ml for extraction. The remaining plasma was pooled (100 ml per batch), extracted with an acid mixture (27) and partially purified with C18 Sep-Pak cartridges [Waters Associates; Millipore (UK) Ltd.] as previously described (24,27) for reverse phase high performance liquid chromatographic (rp-HPLC) studies. For gel permeation chromatography, serum was collected, pooled and lyophilized. Thyroid glands were dissected from rats, wet weights recorded, extracted with acid (27) and partially purified with C18 Sep-Pak cartridges (24,27).

Recovery experiments were carried out after adding a known quantity of synthetic CGRP to plasma, followed by RIA. To estimate the overall recovery of the extraction procedure, unlabeled CGRP or 125 I-CGRP was added to further plasma samples and extracted with acid mixture and partially purified with C18 Sep-Pak cartridges as described above. The former were assayed for i-CGRP by RIA, and in the latter the radioactivity (125 I-CGRP) was counted.

Radioimmunoassay

Antiserum (CG-39) was raised against the C-terminal decapeptide of rat CGRP {[Tyr]⁰rCGRP(28-37), Peninsula} as described previously (11). CC-2/1 antiserum was raised against the intact molecule of synthetic α -hCGRP(1-37) (Sandoz) (6). Epitope mapping with a number of CGRP peptide fragments confirmed that antiserum CG-39 recognized all C-terminal fragments of CGRP (larger than 31-37) and the intact molecule, while antiserum CC-2/1 recognized only the intact CGRP molecules. The

cross-reactivity of the latter with other N-terminal, C-terminal and mid-molecular peptide fragments was <1%. Both antisera fully cross-reacted with α - and β -hCGRP and rCGRP. RIA of plasma and plasma extracts was carried out in two dilutions in duplicate using rCGRP(1-37) as a standard, as described previously (24,27). Sensitivity was 1.0 and 2 fmol/tube for the extraction assay and the direct plasma assay, respectively. At ED₅₀, the intra- and interassay variations were 6% and 9% for the extraction assay and 7% and 11% for the plasma assay, respectively.

Gel Permeation Chromatography

Pooled rat serum (40 ml) was lyophilized and reconstituted in 10 ml of 0.2 mol/l ammonium acetate (pH 5.5), and centrifuged at 4000 × g for 20 minutes at 4°C. The supernatant was loaded (3 ml/chromatographic run) onto a Sephadex G-50 superfine column 1.5 × 85 cm (Pharmacia) and eluted at 4°C with the same buffer. Corresponding fractions from each chromatograph were pooled and lyophilized. Prior to RIA, lyophilized material was dissolved in 30 μ l of 10 mmol/l acetic acid and neutralized with 450 μ l of 50 mmol/l TRIS/HCl containing 0.25% of heat-inactivated BSA (assay buffer). K_{av} values were used for the direct comparison of the elution positions of different molecular forms of CGRP-like immunoreactivity (10). The mean results of three experiments are shown in Fig. 5A.

HPLC

C18 Sep-Pak extracts of plasma were redissolved in 1 ml of 28% acetonitrile in aqueous 0.15% TFA (v/v), 450 μ l was loaded per chromatograph into a Spherisorb ODS 5 μ m, 0.46 × 25 cm column and eluted with a linear gradient up to 44% acetonitrile in aqueous 0.15% TFA over 80 minutes. Corresponding fractions (1 ml) were pooled from each chromatography, lyophilized and assayed for i-CGRP as described above (Fig. 5B). A further batch of 100 ml of rat plasma was extracted similarly after adding ¹²⁵I-rCGRP (100,000 cpm) to assess recovery during extraction of a large volume of plasma. Following extraction with an acid mixture (24,27) and C18 Sep-Pak purification (as in the previous experiment), the recovery of the added label was 78%.

RESULTS

Displacement curves obtained with C18 Sep-Pak extracted and unextracted plasma compared with those of synthetic rCGRP are shown in Fig. 1. i-CGRP could be detected in all plasma samples. When extracted with acid and C18 Sep-Pak cartridges, measurable i-CGRP was only 35% of that of the unextracted plasma. In rats weighing 200 g, the mean i-CGRP value for unextracted plasma (n=32) was 105 ± 11 pmol/l, and for the extracted plasma the mean value was 35 ± 2.8 pmol/l (Fig. 2). However, when synthetic CGRP(1-37) was added to 4 ml of plasma, extracted and partly purified with C18 Sep-Pak cartridge, the recoveries were 84 ± 1.7 and 83 ± 1.3 (n=8 each experiment) for unlabeled and labeled ¹²⁵I-CGRP, respectively. Regression analysis of i-CGRP in 186 of the unextracted and 108 of the extracted rat plasma samples revealed that the level of i-CGRP in plasma was positively correlated (r=.88 and r=.86, respectively) with the age of the rat (p<0.001) (Fig. 2). Among the oldest rats (weight >450 g, age >52 weeks) both plasma i-CGRP levels and the thyroïdal contents of i-CGRP were found to be markedly higher than other groups (Figs. 2 and 4).

The i-CGRP levels of the thyroïd gland (n=98), when ex-

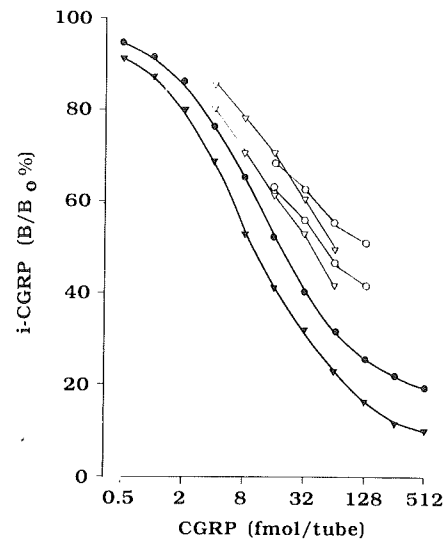


FIG. 1. Standard curves of ¹²⁵I-CGRP obtained with antiserum (CG-39). Displacement of ¹²⁵I-CGRP by synthetic rCGRP(1-37) and/or [¹²⁵I]CGRP(28-37) fragment, using (●) phosphate buffer and (▼) blank plasma. Displacement of ¹²⁵I-CGRP by rat plasma extracts (▽) and unextracted plasma (○).

pressed as pmol/g wet weight or pmol/thyroid gland, also proved to be positively correlated with the plasma i-CGRP (r=.93 and r=.95, respectively, p<0.001) (Fig. 3). High circulating levels of i-CGRP were detected in rats with a higher concentration of i-CGRP in the thyroïd gland, especially in the older rats. In ad-

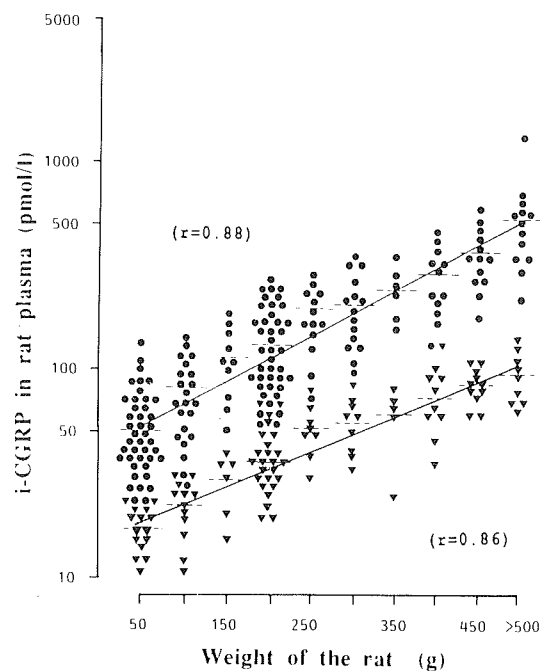


FIG. 2. The correlation of i-CGRP in unextracted (n=186) (●) and C18 Sep-Pak extracted (n=108) (▼) plasma, and the weight of the rats (p<0.001). Linear regression analysis showed a significant correlation between weight of the rat and unextracted plasma (r=.88) and plasma following extraction with C18 Sep-Pak cartridges (r=.86) (p<0.001).

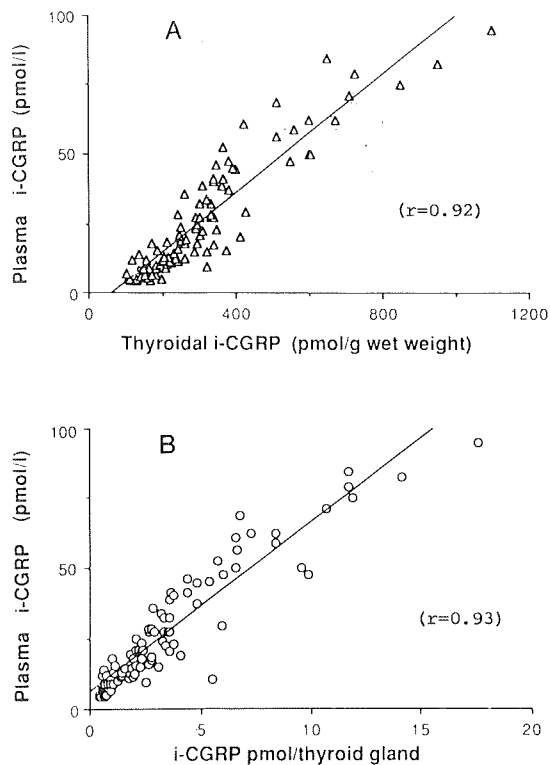


FIG. 3. The correlation of i-CGRP in C18 Sep-Pak extracted plasma and thyroidal content of i-CGRP ($n=98$). The i-CGRP contents are expressed as: (A) pmol/g wet weight (Δ) ($r=0.92$) and (B) pmol/thyroid gland (\circ) ($r=0.93$). Significant positive relationships are shown with plasma i-CGRP and thyroidal contents of i-CGRP ($p<0.001$).

dition, i-CGRP contents in the thyroid glands were highly correlated with the weight (age) of the rat ($p<0.001$) ($r=.88$, pmol/g wet weight; $r=.95$, pmol/thyroid gland, respectively) (Fig. 4). The highest concentration of i-CGRP was found in the thyroid glands of the oldest (>52 weeks) and the heaviest rats.

Gel permeation chromatography of rat serum revealed multiple molecular forms of i-CGRP (Fig. 5A), and only 35–40% of the total immunoreactivity was coeluted when checked with the synthetic rCGRP(1–37). In addition to the monomeric form of CGRP, there were three higher mol.wt. i-CGRP peaks recognized by both antisera and one smaller mol.wt. peak recognized only by specific antiserum to C-terminal fragment of CGRP (CG-39). When plasma extracts were subjected to rp-HPLC and the resultant chromatographic fractions were assayed with antiserum CG-39, there were three relatively hydrophilic immunoreactive peaks (Fig. 5B) in addition to the major i-CGRP peak coeluted with the synthetic rCGRP(1–37) (45%).

DISCUSSION

Unextracted plasma contains three times higher levels of i-CGRP in adult rats (200 g) compared to man (6). The i-CGRP levels measured with the extracted rat plasma, however, are comparable to the previously reported values in the rat (31) and in man (6). The present study shows a significant positive correlation between i-CGRP in both extracted and unextracted plasma with different stages of extra-uterine development of the rat ($p<0.001$) (Fig. 2).

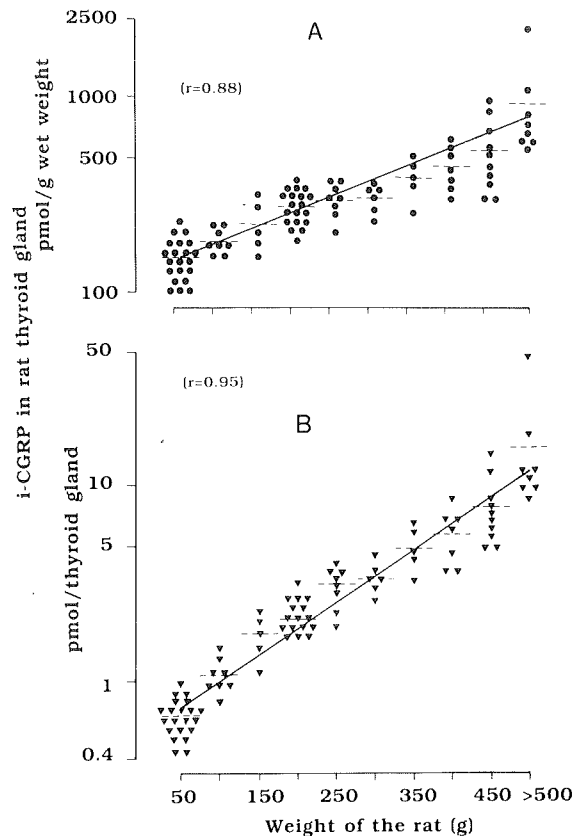


FIG. 4. The correlation of weight of the rat (g) and thyroidal content of i-CGRP: (A) expressed as pmol/g wet weight (\bullet) and (B) pmol/thyroid gland (\blacktriangledown). Linear regressions analysis showed a significant positive relationship between the content of i-CGRP in the thyroid gland and the weight of the rat ($p<0.001$).

Gel permeation chromatography of plasma revealed that only a third of the total i-CGRP was coeluting with synthetic rCGRP(1–37) (Fig. 5A). Furthermore, in rp-HPLC studies of plasma extracts, only 45% of the total immunoreactivity coeluted with the synthetic CGRP(1–37) (Fig. 5B). Therefore, this study revealed that the monomeric form of CGRP (presumably the active form) in the circulation of the rat is only about 30% of the total immunoreactivity.

The higher mol.wt. forms demonstrated in this study could be precursor molecules, molecular aggregates or CGRP bound to a specific (e.g., solubilized receptors) or nonspecific serum binding protein. The smallest molecular weight form of i-CGRP was detected only with antiserum CG-39 and is likely to represent the C-terminal fragment of CGRP. The difference of total i-CGRP detected in the extracted and unextracted plasma, although unlikely, could partly be due to some interference in the unextracted plasma, or due to the larger molecular weight forms or protein bound CGRP which is not retained by the C18 Sep-Pak cartridges (25). As the recovery of added CGRP to plasma prior to extraction was >80%, this further suggests that the latter is the case. A similar phenomenon has previously been shown with calcitonin (CT) (1,8).

Multiple mol.wt. forms of i-CGRP are present in the extracts of the thyroid glands and spinal cord, but these do not contain the highest mol.wt. peak (>50,000 daltons) as seen with plasma

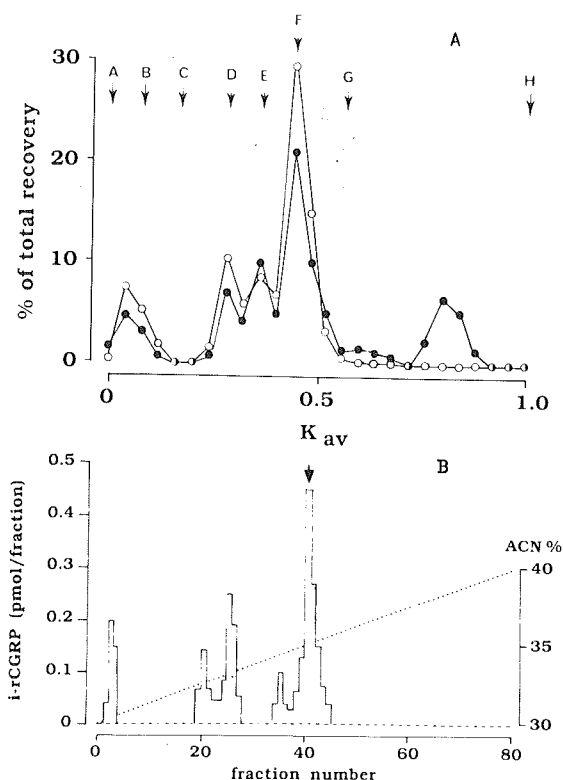


FIG. 5. (A) Gel permeation chromatographic profiles of i-CGRP in rat plasma. Chromatographic fractions were measured with RIA using antiserum CC-2/1 (○) raised against the intact molecule of CGRP(1-37), and antiserum CG-39 (●) raised against a C-terminal fragment of CGRP(28-37). The results were expressed as the percentage of the total yield of i-CGRP after chromatography. (A) Dextran blue (mol.wt. 2×10^6 , V_0); (B) trypsinogen (mol.wt. 24,000); (C) lactoglobulin (mol.wt. 18,400); (D) horse heart cytochrome c (mol.wt. 12,400); (E) aprotinin (mol.wt. 6500); (F) synthetic rat CGRP (mol.wt. 3900), (G) human calcitonin (mol.wt. 3200) and (H) NaCl (salt volume, V_s). (B) rp-HPLC profile (5 μ m ODS, 0.46 \times 25 cm column) of i-CGRP in rat plasma extracts. (\downarrow) = elution position of synthetic rCGRP(1-37).

(27). However, some of the mol.wt. forms present in plasma are likely to have originated from these tissues (22,27). These mol.wt. forms in plasma, as well as in the thyroid gland, do not dramatically change during ageing except for the increase of i-CGRP peak corresponding to mol.wt. 12,500. This confirms that the contribution of i-CGRP from the thyroid gland to the circulation, indeed, increases with age. We have also recently demonstrated the presence of multiple mol.wt. forms of i-CGRP in plasma of both healthy volunteers and in patients with MTC (22).

It has been shown that a major portion of plasma CGRP derives from the perivascular nerves (30), and an increase of plasma i-CGRP and thyroidal CGRP was found in the old rats (31). The present study shows that there is a positive relationship of plasma i-CGRP with thyroidal CGRP which even extends throughout the extra-uterine development of the rat (Fig. 4).

C-cells of the thyroid may undergo hyperplasia and this may contribute to the high incidence of MTC as reported in the ageing rats (2). Whether this is a true tendency or due to the "abnormal diet" received by the laboratory rats, thus causing C-cell hyperplasia, remains to be resolved. Several authors have also reported an age-related increase of plasma immunoreactive CT levels (both basal and stimulated levels) in the rat (9,13). Furthermore, CT has been shown to coexist with CGRP in the C-cells of the thyroid (7) and to cosecrete from cultured MTC C-cells (15). In man, i-CGRP in plasma also increases with advancing age (personal observations). Therefore, it is likely that not only the proportion of CGRP, but also the total quantity liberated from the thyroid to the circulation, may also increase with age.

CGRP is a potent vasodilator, but the physiological significance of circulating CGRP or the increased levels of i-CGRP associated with age (both in plasma and in the thyroid gland) is uncertain. Although a relative deficiency of CGRP has been implicated as a causative factor in Raynaud's phenomenon in man (4,17), its excess so far has not been correlated with disease except for MTC. A wide distribution of i-CGRP and its receptors in the cardiovascular system (26) and i-CGRP in the perivascular nerves (14) suggests that it is likely to have a role in the control of local blood flow, through neural release of CGRP. It is tempting to postulate that the rigidity of the vasculature or the down-regulation of vascular receptors for CGRP with age may act as a stimulus to further secretion of CGRP, thereby increasing the plasma levels. Other possibilities of an increase of CGRP with age are an increase in the percentage of CGRP bound to serum proteins (e.g., increase in serum CGRP binding protein) or a decrease of the rate of metabolism and/or clearance of CGRP from the circulation, and increased synthesis and release of CGRP from other cells. Similarly, in spite of the raised plasma immunoreactive CT in patients with C-cell hyperplasia and in MTC, patients do not present with biological effects of excess CT, such as hypocalcemia or hypophosphatemia. This phenomenon is most likely due to the down-regulations of both renal and bone receptors for CT, as CT extracted from plasma (and/or MTC tissues) is biologically active (12,32).

This study, in addition to demonstrating the immunochemical heterogeneity of CGRP in normal rat plasma, also shows that the monomeric form of CGRP is only about a third of the total circulating i-CGRP. Furthermore, the total i-CGRP measured by RIA depends on the recognition of these multiple immunochemical forms (specificity), and their affinities to the antiserum used. We have previously shown that these problems can be minimized by using an assay based on native receptors, which recognize only the intact mature CGRP (21). This assay has been successfully applied for the estimation of CGRP in a number of physiological and pharmacological studies (28), including the demonstration of a diurnal variation of circulating CGRP in man (23). The relationship of both thyroidal and plasma i-CGRP with age is striking ($p < 0.001$), but its physiological significance remains to be elucidated.

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