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Presence of Immunoreactive Glucagon in Healthy and Diseased Human Thyroid. Evidence of Glucagon Synthesis by this Gland

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A. MENENDEZ-PATTERSON and E. BLAZQUEZ. *Presence of Immunoreactive Glucagon in Healthy and Diseased Human Thyroid. Evidence of Glucagon Synthesis by this Gland.* Rev. esp. Fisiol., **40**, 205-212, 1984.

Significant amounts of immunoreactive glucagon (IRG) were determined in acid-ethanol and acid-saline extracts of human thyroid. Glucagon content of healthy thyroid, expressed as ng/g wet tissue or pg/mg protein, was significantly greater after an acid-alcohol extraction than after an acid-saline one. Furthermore IRG in acid-alcohol extracts of healthy tissue was greater than in acid alcohol extracts of diseased thyroid, while with an acid-saline procedure glucagon content was greater in the extracts of pathological tissues. No significant differences in the IRG content between calcified or follicular thyroid nodules and nodular goiter were found. Aliquots of the tissue extracts, fractionated on Bio-Gel P-30 or Sephadex G-100 columns, gave a 3,500 mol wt immunoreactive peak suggesting the existence of a polypeptide with the same size and immunological properties as pancreatic glucagon. Also, active glucagon synthesis by pieces of thyroid was established by the incorporation of L^3-H-tryptophan into a 3,500 mol wt polypeptide with specific immune reaction to 30K antiserum. These results suggest that thyroid gland could represent a source of extrapancreatic glucagon in men, and therefore contribute to the circulating levels of this hormone.

Key words: Thyroid gland, Human, Glucagon content, Glucagon synthesis.

Until 1974, glucagon was considered to be exclusively a product of pancreatic islets. Then MATSUYAMA and FOÁ (8), and VRANIC, *et al.* (22) reported a persistent glucagonemia in totally depancreatized dogs; SASAKI *et al.* (18), one year later, purified from the gastrointestinal mucosa a polypeptide with properties identical to those of pancreatic glucagon. Since then cells with ultrastructural characteristics similar to pancreatic A cells have been identified in the gastrointestinal mucosa of humans and laboratory animals (1, 12), these cells secrete large amounts of

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glucagon in response to specific stimuli (3, 4, 11). In addition, a broad distribution of glucagon has been found in several other extrapancreatic organs (16, 17). It is interesting to note that significant amounts of glucagon were found in acid-ethanol extracts of rat thyroid (16), and that active glucagon synthesis by slices of this organ was established by the incorporation of L-^{3}H-tryptophan into a 3,500 mol wt polypeptide with specific immune reaction to 30K antiserum. The present study was designed to determine, therefore, if the human thyroid might also be a site of glucagon synthesis and storage. In addition, the thyroid glucagon content was studied in patients with different thyroid diseases and the results obtained were compared with the concentrations found in pieces of healthy tissue.

Materials and Methods

Processing of human thyroid. Pieces of human thyroid were obtained under general anaesthesia from patients undergoing surgery for a variety of thyroid pathologies, such as calcified or follicular thyroid nodules and nodular goiter. Pieces of non-affected glandular tissue surrounding the diseased tissue, as well as pieces of diseased tissue, were selected for study. Most of the patients were women of 35 to 53 year old; only in two cases tissue specimens were obtained on the surgery room from men of 47 and 50 year old. Clinical diagnostic of the patients was confirmed by anatomopathological studies.

The studies were perfomed in accordance with the Helsinki Declaration.

Hormonal extraction and measurement of glucagon. Immediately after surgical removal, small pieces of human thyroid were weighed and homogenized in acid-alcohol solution (ethanol, distilled water, HCl; 75:23.2:1.8, v/v) or in 0.154 mol/l NaCl acidified with HCl to pH 2.8. The homogenates were kept at 4° C overnight, and the precipitates obtained after centrifugation (1,500 *g* for 15 min) were extracted again for 4 h at 4° C. The mixed supernatants were lyophylised and subsequently dissolved in the assay buffer. Samples were preserved frozen until analyzed, whitin of a time in which glucagon content of the frozen samples remains stable. Aliquots of these solutions were used for glucagon determinations as well as for column chromatography. Glucagon was measured by a radioimmunoassay (7), adapted to the use of pork glucagon standard (Novo Research Institute, Copenhagen, Denmark) and a C-terminal reactive antiglucagon serum (30K) generously donated by Dr. R. H. Unger. In this assay, the reactants were mixed as follows: 0.6 ml of 0.2 mol/l glycine buffer pH 8.6, supplemented with 0.25 g/100 ml bovine serum albumin (BSA) and 1 % (v/v) sheep serum, containing mono-^{125}I-glucagon (15 pg) with Trasylol (1,000 KIU/ml), plus 0.2 ml of known (standard curve, from 0 to 2,000 pg/ml) and unknown (experimental samples) glucagon samples and 0.4 ml of 30K antiglucagon serum (1:60.00 final dilution). After an incubation period of 4 days at 4° C, bound and free hormone were separated with dextran coated charcoal (0.5 ml of a 0.5 % charcoal and 0.25 % dextran suspension) in 0.2 mol/l glycine buffer, pH 8.6. Then, the tubes were incubated at 4° C for 45 minutes and centrifuged at 4° C for 15 minutes and the supernatant aspirated by suction. The tubes containing the charcoal pellets were then counted in a gamma scintillation counter. Sensitivity of the assay was up to and including 20 pg/ml and the percentage of intra-and inter-assay variation was 3 % and 6 % respectively.

Gel filtration column chromatography. Aliquots of the acid-saline extracts obtained as described above were further purified by Bio-Gel P-30 or Sephadex G-100 filtration after equilibration of the columns with 0.2 mol/l glycine buffer pH 8.6, supplemented with 0.25 g/100 ml bovine serum albumin (BSA) and 1 % (v/v) sheep serum. Routine column calibration was with blue dextran, ^{125}I-insulin and ^{125}I-glucagon. The immunoreactivities of the eluates were assayed with 30K antibody, as described above.

Synthesis of glucagon from human thyroid gland. Synthesis of glucagon was studied using pieces of human thyroid obtained during surgery. Subsequently, these pieces of tissue (50-100 mg) were incubated with 2 ml of Krebs Ringer bicarbonate enriched with BSA (1 g/100 ml), glucose (100 mg/100 ml), Trasylol (1,000 KIU/ml) and 50 μCi of L-^{3}H-tryptophan (6 Ci/mol; Radiochemical Centre) in an atmosphere of 95 % O_2 and 5 % CO_2 at 37° C, using a metabolic incubator. At the end of the incubation period 1 mg of unlabelled tryptophan was added and the pieces of tissue were homogenized in their own incubation medium. Homogenates were precipitated with trichloroacetic acid (10 %, final concentration) and the resulting sediment extracted with an acid-alcohol solution. Hormonal components were purified further by chromatography in Bio-Gel P-30 columns (1 × 60 cm), precalibrated as described above. The columns were eluted with 0.2 M glycine buffer, containing BSA (0.25 g/100 ml), sheep serum (1 g/100 ml), pH 8.6 and 1 ml fractions were collected. Aliquots of the eluates were taken for measurements of 30K immunoreactivity. For this purpose, aliquots of the eluates were preincubated for 1 h at 37° C with 30K antiserum and subsequently incubated for 48 h at 4° C in the presence of a goat antirabbit gamma globulin. The samples were spun for 15 min at 1,500 *g* and the precipitate was solubilized with 0.01 N NaOH, mixed with 10 ml of the scintillation fluid and counted in a well-type counter.

Results

Immunoreactive glucagon content of healthy and diseased human thyroid. The glucagon content (expressed as ng/g wet tissue and as pg/mg protein) of healthy human thyroid, as determined with 30K antiserum, was significantly greater ($p < 0.05$) after acid-alcohol extraction than after acid-saline extraction (table I). Also, the hormonal content in acid-alcohol extracts of healthy thyroid tissue was significantly greater ($p < 0.05$) than in acid-alcohol extracts of diseased thyroid tissue. In contrast,

Table I. *Immunoreactive glucagon content in samples of healthy and diseased human thyroid extracted with acid-ethanol or acid-saline solutions.*
Means ± SE. N = 5-7. The hormone was determined as described in Methods.

	GLUCAGON CONTENT			
	Acid-ethanol		Acid-saline	
	Wet tissue ng/g	Protein pg/mg	Wet tissue ng/g	Protein pg/mg
Healthy tissue	6.21 ± 0.44	54.65 ± 39.90	1.68 ± 0.07	8.31 ± 0.85
Diseased	1.50 ± 0.52	8.83 ± 0.45	3.20 ± 1.13	18.17 ± 5.76

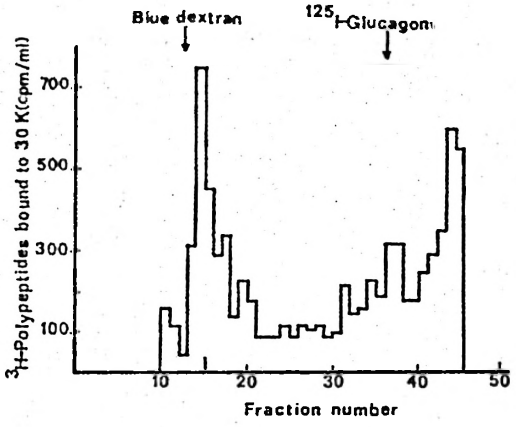

Fig. 1. *Gel filtration of acid-saline extract of a follicular thyroid nodule.*
The material applied to columns (1 × 60 cm) of Bio-Gel P-30 (100-200 mesh) was equilibrated with 0.2 mol/l glycine buffer, pH 8.6, plus 0.25 g/100 ml BSA; 1 ml fractions were collected; aliquots were assayed for IRG with 30K antiserum.

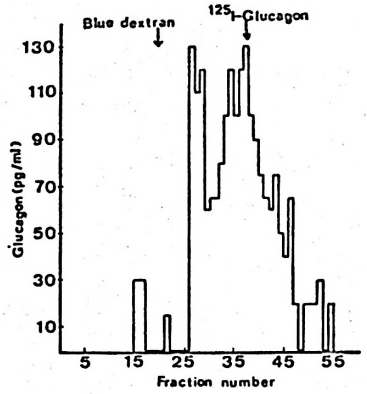

Fig. 2. *Gel filtration of acid-saline extract of healthy human thyroid.*
The material applied to columns (1 × 60 cm) of Sephadex G-100 was equilibrated with 0.2 mol/l glycine buffer, pH 8.6, plus 0.25 g/100 ml BSA; 1 ml fractions were collected; aliquots were assayed for IRG with 30K antiserum.

the amount of glucagon extracted from diseased thyroid tissue (calcified or follicular thyroid nodules and nodular goiter) was greater with an acid-saline solution than with an acid-alcohol one. No significant differences in the IRG content between calcified or follicular thyroid nodules and nodular goiter were found.

As we have reported previously (16), the percentage of glucagon degradation during the acid-ethanol hormone extraction was minimal, even in the absence of Trasylol (1,000 KIU/ml). However with the acid-saline extractions procedure there was 67.3 % glucagon degradation; even with Trasylol present, there was still 48 % degradation.

Characterization of immunoreactive glucagon (IRG) in normal and diseased human thyroid extracts. The chromatography profiles of normal and diseased human thyroid extracts are shown in figs 1 and 2. Different peaks of immunoreactivity were detected in both extracts after fractionation on either Bio-Gel P-30 or Sephadex G-100 columns, but one peak in particular eluted with the ^{125}I-glucagon marker in both

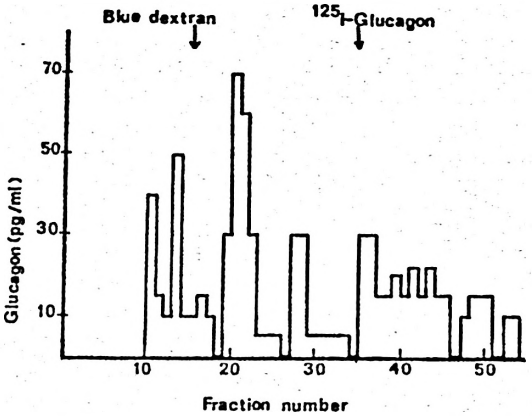

Fig. 3. *Elution profile from Bio-Gel P-30 of proteins from healthy human thyroid labeled with L-^{3}H-tryptophan.*
Aliquots of the eluates were incubated with 30K antiserum.

cases. The proportion of IRG eluting in the same position as ^{125}I-glucagon over immunoreactive molecules of larger molecular weight was greater in extracts of healthy human thyroid than in those of diseased thyroid.

Synthesis of glucagon from samples of healthy human thyroid. Pieces of tissue from normal human thyroid were incubated *in vitro* with L-^{3}H-tryptophan. After 2 h of incubation, the tissue acid-ethanol extract was further purified in Bio-Gel P-30 column. Aliquots of the eluates were incubated with 30K antiserum and the radioactivity bound to glucagon antibody was found (fig. 3) in several peaks, one of which was localized in the same position as ^{125}I-glucagon.

Discussion

In recent years, hormones such as gastrin and vasoactive intestinal peptide have been detected in unexpected locations (5); peptides of neural origin have been found in pancreatic and gastrointestinal endocrine cells (15), rapidly changing our information about the cells that produce and release polypeptide hormones (14). Glucagon, until 1974 considered a synthetic product of pancreatic islets, has since been found in several extrapancreatic organs of experimental animals (6, 16, 17), such as the gastrointestinal tract, salivary glands, thymus, brain and thyroid gland. In humans, too, evidence of the existence of extrapancreatic glucagon has been presented. Although extrapancreatic glucagon seems to be less abundant in man than in experimental animals, glucagon containing cells have been described in the human stomach (12), and significant amounts have been extracted from human salivary glands (17). According to our results, the human thyroid could also contribute to the circulating levels of this hormone, because significant amounts of glucagon have been determined there after acid-alcohol and acid-saline tissue extractions. These glucagon tissue concentrations are slightly smaller than in human submaxillary glands and greater than in human parotid glands (17), but much smaller than in rat thyroid (16). Also, some differences between healthy and diseased human thyroid were found. In fact, after acid-alcohol extraction, hormonal content was greater in healthy tissue, while after acid-saline extraction it was significantly higher in diseased tissue. These findings could be related to the greater ability of acid-saline solution to extract larger glucagon immunoreactive molecules and to the possibly greater proportion of glucagon in diseased tissues. Thus, after chromatographic fractionation we found a greater proportion of larger immunoreactive glucagon molecules than true glucagon in diseased human thyroid extracts as compared with healthy tissue extracts.

Although significant amounts of immunoreactive glucagon have been found in human thyroid using a C-terminal antiserum (30K), the question remains whether the presence of this immunoreactive component indicates active synthesis and storage or merely selective trapping of circulating glucagon by the thyroid gland. Since after chromatographic fractionation of thyroid extracts an immunoreactive peak of 3,500 daltons was always obtained, we suggest that in human thyroid there is a polypeptide of the same size and immunological properties as pancreatic glucagon. Also, active glucagon synthesis by this organ was established by the incorporation of L-^{3}H-tryptophan into a 3,500 molecular weight polypeptide with specific immune reaction to 30K antiserum.

These results seem to indicate that

human thyroid gland represents a source of extrapancreatic glucagon, which could contribute to the circulating levels of this hormone, as it happens for gastrointestinal tract and salivary glands. In support of this statement, several authors have reported normal glucagon concentrations in patients after surgical removal of the pancreas and/or stomach (2, 9, 10, 13, 19-21). Also, it has been described (16), that glucagon release by pieces of rat thyroid is stimulated by arginine, norepinephrine and low glucose concentrations. Nevertheless, more information is needed about the physiological role of human thyroid gland as a contributor to the circulating levels of glucagon, and therefore to metabolic homeostasis.

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Resumen

Se determina glucagón inmunorreactivo (IRG) en extractos ácido-alcohólicos y salino-acidificados de tiroides humano.

El contenido de glucagón en trozos de tiroides sano es significativamente mayor después de una extracción en ácido-alcohólica que en salino-acidificado. El contenido de IRG en los extractos ácido-alcohólicos es superior en el tejido sano respecto al enfermo, mientras que con una extracción salino-acidificada se obtienen resultados contrarios a los anteriores. No se observan diferencias significativas entre las muestras tisulares pertenecientes a nódulos tiroideos foliculares o calcificados y bocios nodulares. Alícuotas de los extractos tisulares fraccionados cromatográficamente en columnas de Bio-Gel P-30 o Sephadex G-100, dan siempre un pico de IRG con peso molecular de 3.500 daltones lo cual indica la existencia en el tiroides humano de un polipéptido con el mismo tamaño y propiedades inmunológicas que el glucagón pancreático. Trozos de tiroides incubados con L-^{3}H-triptófano, incorporan este aminoácido en un polipéptido que reacciona con el antisuero 30K. Estos resultados sugieren que el tiroides humano puede ser una fuente de glucagón extrapancreático que podría contribuir a los niveles circulantes de esta hormona.

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