Calcitonin mRNA activity in young obese (fa/fa) Zucker rats

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Alterations in both calcitonin (CT) secretion and plasma calcium were recently described in adult obese Zucker rats. We have investigated the CT biosynthetic activity of thyroid glands in 30-day-old obese Zucker rats (fa/fa), and their controls (Lean). Plasma calcium level was significantly increased (+0.6 mg/dl) in obese animals, but plasma phosphate was unchanged. Plasma CT levels measured by radioimmunoassay (RIA) were significantly decreased in fatty (0.50 ± 0.03 *vs* 0.68 ± 0.03 ng/ml in Leans; $P < 0.001$), but thyroidal hormone content was not different between Lean and fatty rats (68.7 ± 5.1 in Leans *vs* 60.5 ± 3.6 ng/gland in fatty rats). mRNA was extracted from 10 thyroids, and translated in a rabbit reticulocyte lysate (NEN) in the presence of [^{35}S]methionine. After polyacrylamide gel electrophoresis, specific immunoprecipitates were autoradiographied and quantified by integration. A 50% decrease in translatable CT mRNA was observed in fatty rats. In basal conditions, the biosynthetic activity of C cells in obese rats correlates with the secretion rate of the hormone in the face of unchanged thyroidal CT contents.

mRNA activity *Calcitonin* *Young obese rat* *Zucker rat*

1. INTRODUCTION

Calcitonin (CT) is a hormone involved in the regulation of calcium metaboism, but little is known about the regulatory processes implicated in its synthesis and secretion. It has been shown that the hormone is synthesized in the form of a large precursor (M_r = 15000) [1–5] of established sequence [6,7]. The presence of alterations in CT secretion in genetically obese rats has been described [8,9], but the cause remains obscure. The Zucker fatty rat (fa/fa) is an animal model for obesity in which fattiness is inherited as a single autosomal-recessive gene of unknown function [10]. A possible relation between a change in CT biosynthesis and the altered CT secretion prompted us to evaluate the biosynthetic activity of C cells in young Zucker rats. Thus, CT mRNA activity of thyroid glands was analysed in fatty animal (fa/fa) and their controls (Lean) using the relative abundance of mRNA [11] measured by translation assay.

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2. MATERIALS AND METHODS

2.1. *Animals*

Thirty-day-old fatty (fa/fa) Zucker rats and their controls (Lean) were killed by decapitation; thyroid glands were removed and stored in liquid nitrogen until extracted.

2.2. *mRNA extraction*

Ten thyroid glands were pooled and homogenized with a Polytron in 1 ml of 0.1 M Tris–HCl, 0.01 M EDTA (pH 9) buffer (Tris–EDTA buffer) containing 0.1 M dithiothreitol, 2% SDS and 1 ml of phenol–chloroform–isoamyl alcohol (50:50:2) previously equilibrated with Tris–EDTA buffer. Extracts were cooled in ice and centrifuged at 6000 rpm for 10 min. The organic phase was re-extracted with an equal volume of Tris–EDTA buffer. Combined aqueous phases were extracted

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3 times with an equal volume of phenol–chloroform–isoamyl alcohol solution. Total nucleic acids were precipitated by alcohol in the presence of 0.1 vol. of 2 M sodium acetate (pH 5.5) at −20°C for 16 h. The precipitates collected by centrifugation were washed with cold 75% ethanol and recentrifuged. The total nucleic acids were dissolved in distilled water and precipitated by treatment with 0.25 vol. of 10 M LiCl overnight [12]. Poly(A)-rich RNA was separated by affinity chromatography using oligo(dT)–cellulose T3 (Collaborative Research) [13].

2.3. *Translation studies*

Aliquots of mRNA extracted from 10 thyroids were dissolved in sterile distillated water.

Reticulocyte lysate: 1 μg mRNA (in 2 μl water) was added to 20 μl rabbit reticulocyte lysate obtained from New England Nuclear (NEN) containing 50 μCi [^{35}S]methionine (NEN) and incubated for 60 min at 37°C.

Radioactivity incorporated into synthesized proteins was estimated by precipitation with trichloroacetic acid [14]: 1-μl aliquots were spotted on 3MM Whatman paper wetted with 20% trichloroacetic acid, boiled for 10 min in 1 l of 10% trichloroacetic acid, dried with diethyl ether, and counted. For immunoprecipitation, 10- or 12-μl aliquots were incubated for 24 h at 4°C in 300 μl of 0.1 M phosphate buffer (pH 7.4) containing 0.5% Triton X-100, 0.2% human albumin, 0.1% methionine in the presence of 0.7 μl sheep antiserum (M 732) against human synthetic CT saturated or not with 10 μg human CT. Immunoglobulins were then precipitated by the addition of 25 μl anti-sheep (IgG) for 24 h at 4°C. Precipitates were collected by centrifugation over 200 μl of a 1 M sucrose cushion in a 1.5 ml polypropylene tube (Eppendorf), and washed 5 times with the immunoprecipitation buffer. Precipitates were dissolved and heat-denatured in 25 μl of 60 mM Tris (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol and 0.4% bromophenol blue. Aliquots were counted in scintillation mixture (Biofluor, NEN), specificity of the immunoprecipitates was checked by 0.1% SDS-polyacrylamide gel electrophoresis (SDS–PAGE) (20% acrylamide) [15] and autoradiography performed using Kodak X-Omat AR5 film (8-day exposure at −80°C). Autoradiographies were quantified in an automatic densitometric scanner.

2.4. *Other methods*

Blood samples were collected in polyethylene tubes cooled in ice water. After centrifugation at 4°C, aliquots of plasma were used immediately for calcium and phosphorus determinations, the remainder being frozen (−35°C) until CT was assayed. The thyroid glands were dissected under a microscope at 4°C and sonicated (MSE 150 W Ultrasonic Desintegrator Mk2, Crawley, England) at 4°C for 30 s in barbital buffer (0.025 M, pH 8.6) containing peptidase inhibitors [16].

The CT in plasma and sonicates was measured by a radioimmunoassay (RIA) which has been described [17]. The plasma calcium concentration was measured by flame photometry (Eppendorf, FCM 6341), and the plasma phosphate level was evaluated as in [18].

Results were expressed as means ± SE. Significance of differences between groups was determined using Student's *t*-test.

3. RESULTS AND DISCUSSION

Plasma calcium levels were increased by 0.61 mg/dl in 30-day-old obese animals (fig.1), whereas no difference was found for plasma phosphate. These findings confirm previous observations for adult Zucker rats [8,9]. Plasma CT levels were decreased in fatty rats (0.50 ± 0.03 in fatty rats *vs* 0.68 ± 0.03 ng/ml in Leans; $P < 0.001$), but thyroidal CT contents were unchanged (fig.2). Higher plasma CT levels and increased thyroidal CT contents were reported in pubertal fatty Zucker rats [8,9]. We have obtained similar results after puberty (not shown), but in prepubertal animals 30- or 42-day-old (not shown) decreased plasma CT levels were observed. Since hypercalcemia in fatty Zucker rats is associated before puberty with decreased plasma CT, and after puberty with elevated plasma CT, the shift in plasma calcium is unlikely to be related to the change in CT secretion. The presence of elevated plasma lipoproteins in obese animals may be the cause of this hypercalcemia.

Total RNA extracted from the thyroid glands was slightly higher in Leans (169.2 μg) than in obese (138.6 μg) rats. The same finding was made

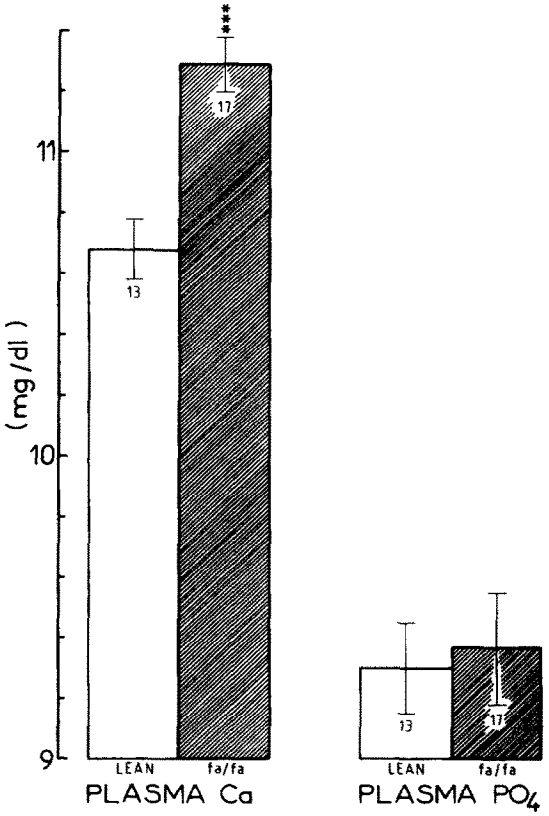

Fig.1. Plasma calcium and plasma phosphate levels in 30-day-old fatty (fa/fa) Zucker rats. Lean, lean littermate controls. Mean ± SE and the number of animals. *** $P < 0.001$ from controls.

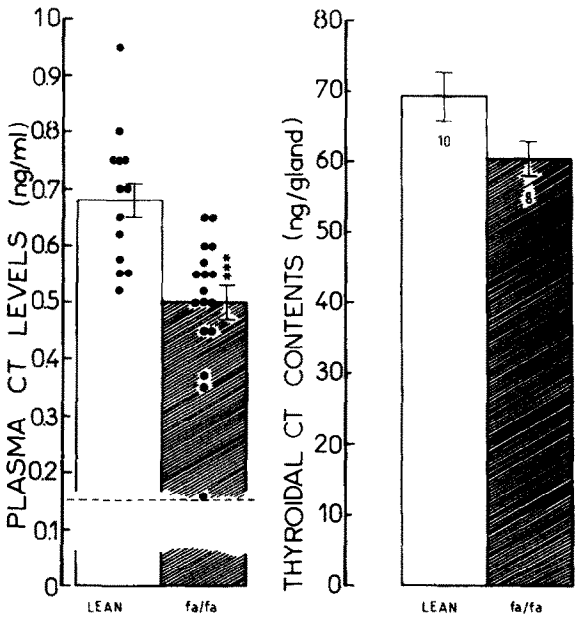

Fig.2. Plasma calcitonin (CT) levels and thyroid CT contents in 30-day-old fatty (fa/fa) Zucker rats. Lean, lean littermate controls. Individual values are given for plasma CT. Mean ± SE and the number of animals. *** $P < 0.001$ from controls.

for total nucleic acids (263.7 μg in Leans *vs* 182.2 μg in fatty rats). Total poly(A)-rich RNA and total proteins synthesized by translation of the mRNA were not different between fatty and Lean animals: 340 μg poly(A)-rich RNA was extracted from 10 thyroid glands of Leans and 3.65 μg in fatty animals. Polyacrylamide gel analysis and autoradiography of the immunoprecipitates showed the presence of a major band (M_r 15000) (fig.3a,4a). This band was absent in immunoprecipitates using specific antiserum saturated with unlabeled CT (fig.3b,4b). This observation in Zucker rats was in agreement with previous data on the M_r of preprocalcitonin [1–5]. Translatable CT mRNA activity was half-reduced in fatty rats when compared to controls (fig.3,4). Thus, 30-day-old fatty rats exhibited both reduced CT biosynthesis and secretion. This decrease is concomitant with a fall in the plasma levels of the hormone, while CT tissue stores were unaffected. This implies that a decrease in the relative abundance of translatable mRNA is associated with the genetic defect of the fatty rat. The decrease in CT mRNA could be due to a drop in transcriptional activity or to post-transcriptional and/or pretranslational level involving the maturation, inactivation of the specific messengers. We have reported that an increase in CT mRNA correlated with an increased CT secretion after acute calcium stimulation [5]. Our results show that in the opposite situation the same relationship exists, further substantiating the presence of direct or indirect regulatory mechanisms coupling gene expression and secretion. Furthermore, this is the first time that a genetically linked alteration in CT biosynthetic activity has been described in obese Zucker rats. Of potential clinical interest is whether alterations in CT biosynthetic activity are directly linked to the genetic defect or are a consequence of the resulting obesity.

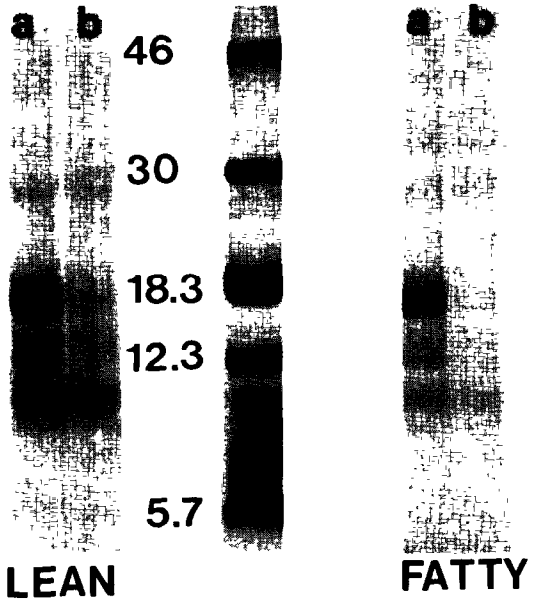

Fig.3. Autoradiographies of specific immunoprecipitates obtained by translation of 1 μg thyroid mRNA. The proteins synthesized and immunoprecipitated were analysed by 0.1% SDS–20% PAGE. (a) Without unlabeled human CT; (b) with an excess of unlabeled human CT (10 μg). Numbers by the middle lane represent $M_r \times 10^{-3}$ of labeled markers: insulin, 5700; cytochrome *c*, 12300; lactoglobulin A, 18300; carbonic anhydrase, 30000; ovalbumine, 46000.

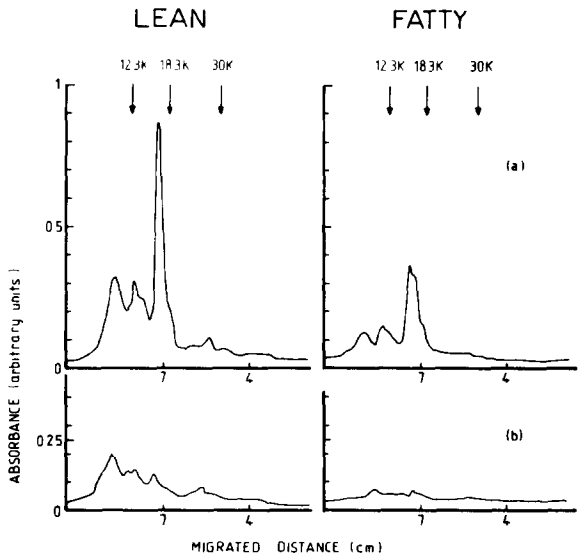

Fig.4. Densitometric scans of autoradiographies shown in fig.3a without unlabeled human CT (b) with an excess of unlabeled human CT (10 μg). K, kDa.

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