Pituitary Neuromedin B Content in Experimental Fasting and Diabetes Mellitus and Correlation With Thyrotropin Secretion

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Fasting and diabetes mellitus in the rat model have been associated with abnormalities of thyrotropin (TSH) secretion. Neuromedin B is a bombesin-like peptide highly concentrated in the pituitary gland that has been shown to have inhibitory action on TSH secretion, acting as an autocrine/paracrine factor. Here, we aimed to determine if the pituitary content of neuromedin B would change in fasted rats (1, 2, 3, and 4 days of food deprivation) and streptozotocin (55 mg/kg body weight)-diabetic rats. The total pituitary content of neuromedin B was decreased in fasted rats, except at 2 days of fasting, as was the total protein content in the gland; however, the concentration of the peptide (femtomoles per milligram protein) did not significantly change until the fourth day of food deprivation, when an abrupt decrease in total protein happened and therefore neuromedin B concentration increased. In rats after 20 days of diabetes induction, pituitary neuromedin B increased. Serum thyroxine (T_4) and triiodothyronine (T_3) decreased in both disorders, whereas serum TSH was normal or decreased in 4-day fasted rats. Therefore, the caloric deprivation of diabetes and fasting changed the pituitary neuromedin B content and concentration, by mechanisms that remain to be elucidated. Since neuromedin B has been shown to act as a local inhibitor of TSH release, the results raise the possibility that increased neuromedin B concentration might be involved in the altered TSH secretion of diabetes mellitus and fasting.

FASTING AND DIABETES MELLITUS are metabolic disorders that display common general abnormalities of the hypothalamus-pituitary-thyroid axis. Several data from experimental models indicate that the synthesis and release of thyrotropin (TSH) are impaired in both disorders. Food-deprived rats and chemically induced diabetic rats have been reported to have normal[1,2] and, more frequently, decreased[3-7] serum TSH, as well as normal or decreased pituitary TSH,[2,8,9,10] and decreased β-TSH and β-TSH mRNA.[7,11] These changes would be unexpected in the face of the decreased serum thyroxine (T_4) and triiodothyronine (T_3) generally present in both disorders.[4,11] It is unsettled as to whether the abnormalities of TSH regulation in fasting and diabetes mellitus are primarily due to pituitary mechanisms leading to an increased sensitivity of thyrotropes to thyroid hormones[3,5,6] or are mainly a consequence of altered input from hypothalamic regulators.[2,7,8,10]

Neuromedin B is a bombesin-like peptide that is highly concentrated in the pituitary gland[12-14] as a result of local synthesis.[15] Neuromedin B immunoreactivity was detected by immunohistochemistry within the thyrotrope in the rat pituitary.[16] Neuromedin B has an inhibitory action on TSH secretion both in vivo and in vitro at physiological doses.[17,18] Moreover, immunoneutralization of neuromedin B induced an increase in TSH secretion not only when the antiserum was injected into normal and hyperthyroid rats, but also when it was present in the incubation medium of isolated pituitaries.[18] Altogether, the data strongly suggest that neuromedin B acts as an autocrine inhibitor of TSH secretion. Neuromedin B synthesis in the pituitary gland is apparently under hormonal control. Thyroid hormones and estrogen positively regulate[15] both the peptide and mRNA content in the gland.

Therefore, since fasting and diabetes mellitus are associated with decreased TSH secretion and pituitary neuromedin B is a local peptide with a postulated physiological role in the inhibition of TSH secretion, we designed this experiment to determine if pituitary neuromedin B content would change in fasted and diabetic rats.

MATERIALS AND METHODS

Animal Treatment

In all experiments, we used adult male rats maintained in a room with controlled lighting (12-hour light/dark cycle, lights on at 7 AM) and temperature (23° to 26°C).

Fasting Protocol

Animals weighing 260 ± 7.8 g were divided into five groups of nine to 11 animals each of similar weight. The animals were food-deprived for 1, 2, 3, or 4 days before being killed. The control group was fed rat chow ad libitum. All animals were decapitated on the same day, and the trunk blood and anterior pituitaries were quickly dissected out and neuromedin B was extracted from the tissue as described later.

Diabetes Mellitus Protocol

Diabetes mellitus was induced by a single intraperitoneal injection of 55 mg/kg body weight streptozotocin (Sigma, St Louis, MO) diluted in no more than 0.5 mL 50-mmol/L citrate buffer, pH 4.5. The control group received the same volume of the vehicle. Blood glucose levels were determined 48 hours after streptozotocin injection and at the end of the experimental period using glucofilm with a glucometer (Ames, Elkhart, IN). Glycemia was measured after 4 hours of fasting. Diabetic animals always had glycemia greater than 350 mg/dL.

Twenty days after diabetes induction, all animals were killed by exsanguination under ether anesthesia, and blood glucose concentrations were immediately determined. Anterior pituitary glands were

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rapidly removed, and neuromedin B was extracted from the glands per the following description.

Quantification of Anterior Pituitary Neuromedin B

Neuromedin B was extracted from anterior pituitaries by boiling (100°C) homogenates prepared in 500 mmol/L acetic acid for 15 minutes. Extracts were centrifuged at 1,600 × *g* for 15 minutes, and the supernatants were then frozen in liquid nitrogen and stored at −70°C until assayed. Neuromedin B level was measured by a radioimmunoassay using a highly specific antiserum that, as described by Namba et al,[16] showed less than 0.05% cross-reactivity with all relevant bombesin-like peptides, with the exception of the amphibian peptides, litorin and ranatensin, which showed 70% and 60% cross-reactivity, respectively. *N*-terminal–tyrosylated neuromedin B-10, synthesized at the Department of Pharmacology of Southwestern Medical School (Dallas, TX), was labeled with ^{125}I (ICN Biomedicals, Costa Mesa, CA) by conventional chloramine-T oxidation. Labeled neuromedin B was purified using carboxymethyl cellulose-52 (microgranular, preswollen; Sigma) cation-exchange chromatography, and free iodide was eluted with 0.01 mol/L ammonium acetate buffer, pH 4.6, and the peptide was eluted with 0.5 mol/L ammonium acetate buffer, pH 4.6. Neuromedin B standards were prepared from pure synthetic Neuromedin B-10 (Sigma). The assays were performed in 60 nmol/L phosphate buffer, pH 6.8, containing 10 mmol/L EDTA, 100 μmol/L bovine serum albumin, and 1.85 mg/L aprotinin (Sigma). Standards or extracted samples were incubated at 4°C with the antiserum at a final dilution of 1:7,000 for 24 hours, and then ^{125}I-labeled neuromedin B (10,000 cpm per tube) was added and the incubation continued for more 4 days at 4°C. The labeled antigen-antibody complex was precipitated with 1 mL ice-cold absolute ethanol, followed by centrifugation at 1,600 × *g* for 20 minutes at 4°C. The supernatant was discarded, and radioactivity in the precipitate was measured. The samples were assayed in duplicate. All samples for each experiment were measured within the same assay. Within-assay variation was 5.3%, and the coefficient of variation between assays was 8.6%. Minimum assay detection was 5 fmol/100 μL.

Neuromedin B values were expressed as total content per gland (femtomoles per gland) or as the concentration corrected for the total protein in the gland (femtomoles per milligram protein). Protein content was determined by the Bradford method.[19]

Quantification of Serum TSH, T_4, and T_3

Blood samples were centrifuged, and the serum was stored at −20°C for T_4, T_3, and TSH determination by specific radioimmunoassays.[20]

TSH was determined with kits supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD) and is expressed in terms of the reference preparation (RP3). TSH was labeled with ^{125}I by the chloramine-T method. Labeled antigen-antibody complex was precipitated using a goat anti-rabbit immunoglobulin G (Sigma) plus polyethylene glycol (6%), followed by centrifugation at 1,600 × *g* for 30 minutes at 4°C. Within-assay variation was 7.9%, and the coefficient of variation between assays was 6.7%. Minimum assay detection was 0.52 ng/mL.

T_4 and T_3 concentrations were measured by radioimmunoassays using specific antiserum purchased from Sigma, and the assays were performed following the manufacturer's instructions. Briefly, ^{125}I-labeled T_4 or T_3 (DuPont-NEN, Boston, MA) at a specific activity of 1,320 μCi/μg and 1,200 μCi/μg, respectively, were incubated with sample or standards (Sigma) for 1 hour. Labeled antigen-antibody complex was precipitated by the double-antibody method plus polyethylene glycol. For the T_4 assay, within-assay variation was 8.9% and the coefficient of variation between assays was 7.2%. For T_3, within-assay variation was 10.6% and the coefficient of variation between assays was 9.2%. The limit of detection was 0.49 μg/dL for T_4 and 0.64 ng/dL for T_3.

Statistics

The data are reported as the mean ± SEM. *P* less than .05 was taken as the level of significance. For the fasting experiments, one-way ANOVA followed by a Student-Newman-Keuls multiple-comparison test was used for assessment of significance of all data except serum TSH, which was analyzed by the Kruskall-Wallis test followed by Dunn's multiple-comparison test. For diabetes mellitus, Student's *t* test was used for assessment of significance of all data except serum TSH, which was analyzed by the Mann-Whitney rank-sum test.

RESULTS

As expected, the severity of caloric deprivation increased with time into the fast, which was reflected by a progressive loss of body weight of fasted rats (Table 1). Total serum T_4 showed an abrupt decrease (~45% of the level in the fed group, $P < .05$) at 1 day of fasting, and a gradual decline occurred over the next 3 days, achieving a value 80% lower than in the fed control group (Table 1). Total serum T_3 decreased abruptly and markedly from 1 day into the fast, reaching less than detectable levels at 3 and 4 days of food deprivation. Despite the decrease in serum T_4 and T_3, serum TSH concentration was maintained normal during the first 3 days of fasting. However, after 4 days of food deprivation, serum TSH decreased to approximately half the concentration of the previous days (Table 1).

The total pituitary neuromedin B content decreased (~20%) in animals food-deprived for 1 day (Fig 1A). Apparently, at 2 days of fasting, some adaptative mechanism prevented the decrease of neuromedin B content. However, after 3 or 4 days into the fast, pituitary neuromedin B content was again decreased about 35%. However, the decreased neuromedin B content seems to be consequent to the general catabolic process induced by caloric deprivation, since total pituitary protein decreased progressively in fasted rats. Therefore, neuromedin B concentration corrected to milligrams of total protein in the gland (femtomoles per milligram protein) actually was unchanged from 1 to 3 days of starvation (Fig 1B). Animals

Table 1. Serum Concentration of T_4, T_3, and TSH and Protein Concentration in the Anterior Pituitary of Fasting Rats

Group	No.	Initial BW (g)	BW Loss (%)	Protein (mg/pituitary)	T_4 (μg/dL)	T_3 (ng/dL)	TSH (ng/mL)
C	9	273 ± 8.0	—	1.73 ± 0.1	3.31 ± 0.3	25.7 ± 1.60	1.02 ± 0.1
F1	11	260 ± 7.8	5.0	1.31 ± 0.1†	1.84 ± 0.3*	2.1 ± 0.80†	1.02 ± 0.1
F2	10	274 ± 7.3	9.1	1.14 ± 0.1†	1.23 ± 0.1*	0.7 ± 0.04†	1.15 ± 0.1
F3	10	268 ± 9.9	11.2	1.14 ± 0.1†	0.87 ± 0.2*	<0.64†	1.23 ± 0.1
F4	9	263 ± 8.6	19.0	0.61 ± 0.1†	0.60 ± 0.1†	<0.64†	0.64 ± 0.1*

Abbreviations: F1 to F4, rats fasted for 1 to 4 days; C, control rats; BW, body weight.

*$P < .05$.

†$P < .001$.

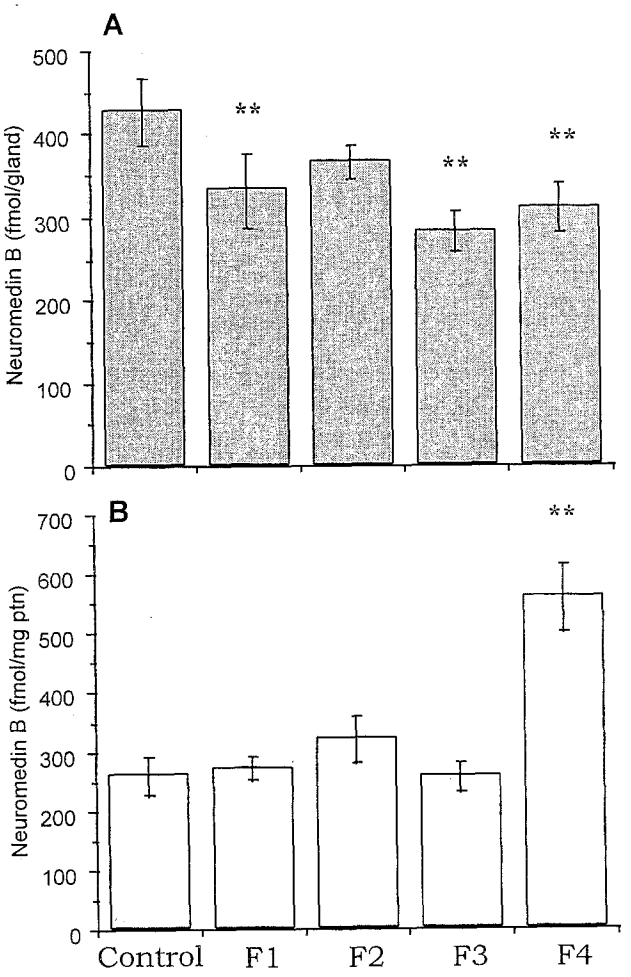

Fig 1. Anterior pituitary neuromedin B content expressed as fmol/gland (A) or fmol/mg protein (B) in rats fasted for 1 to 4 days (F1 to F4) or fed ad libitum (control). **$P < .001$.

submitted to food deprivation for 4 days showed a marked decrease (~65%) in total protein in the gland, which was not accompanied by the same magnitude of decrease in neuromedin B; consequently, the concentration of neuromedin B significantly increased (~twofold, $P < .001$) in relation to the other groups.

Rats killed 20 days after induction of diabetes mellitus by streptozotocin treatment showed, as expected, high levels of glycemia and a 25% decrease in body weight. Responses regarding thyroid indices were similar to those of fasting rats, although there were differences in magnitude (Table 2). Serum T_3 was importantly decreased in diabetic rats ($P < .001$), whereas serum T_4, although lower, did not differ significantly from that of the control group. Serum TSH concentration showed a tendency to decrease, but it did not reach statistical significance ($P = .06$).

Pituitary neuromedin B (Fig 2) expressed either as total content or as concentration showed a small (15% to 25%) but significant increase ($P < .05$) compared with control levels, regardless of the small decrease in total protein content in the gland (Table 2).

DISCUSSION

The decreased pituitary neuromedin B content of fasted rats might not be due to a specific mechanism, but rather to the catabolic state, as suggested by the parallel decrease in total protein in the gland. However, the severity of caloric deprivation, especially after 4 days of food deprivation, did not affect neuromedin B as much as other proteins in the gland, and therefore, from 1 to 3 days into the fast the concentration of pituitary neuromedin B remained unchanged, whereas after 4 days of food deprivation it actually increased. Also, in streptozotocin-induced diabetic rats, the concentration of the peptide increased, as well as the total content in the gland, albeit with a small decrease in protein content.

In the present study, serum T_4 and T_3 were reduced since the first day of fasting, whereas serum TSH was maintained normal until the fourth day into fasting. Some investigators[11,21] postulated that the decrease in serum T_4 is consequent to the reduction in serum TSH. However, others[3,22] could not find any change of serum TSH in fasting rats, despite the presence of a significant reduction in serum T_4 and T_3. The reason for this controversial result is not clear. It is possible that other mechanisms besides a reduction in serum TSH contribute to the reduction in serum T_4, especially in the early phases of food deprivation. It had been proposed that TSH bioactivity might be altered in fasting rats,[22] since during hypothalamic hypothyroidism the TSH carbohydrate structure is altered.[23] Another possible mechanism involves a reduction in thyroid responsiveness to TSH in fasting, as we suggested previously, since long-term (1 to 4 days) TSH administration to fasting rats did not restore serum levels of thyroid hormones.[1]

The mechanisms for the changes in pituitary neuromedin B are not evident from the present study. As expected, in diabetes mellitus and fasting, serum TSH failed to show a compensatory increase in the presence of low thyroid hormone levels. On the contrary, after 4 days of food deprivation, the rats showed an abrupt decrease in serum TSH that was coincidental with the lowest serum T_4 and T_3. Also, the findings concerning pituitary neuromedin B are not expected, since such low levels of thyroid hormones, mainly like those observed in fasting rats, were supposed to induce a much more pronounced decrease in the peptide content of the pituitary gland. In hypothyroid rats, pituitary neuromedin B content is severely decreased,[14,15] probably due to reduced synthesis, as suggested by decreased levels of neuromedin B mRNA.[15] Therefore, other factors might be involved in the changes in neuromedin B concentration in those metabolic disorders. It has been suggested that in fasting and diabetes mellitus, there is an increased sensitivity of the pituitary gland to the suppressive effects of thyroid hormones on TSH.[3,5,6] However, responsiveness to thyroid hormones was found to be decreased in relation to growth hormone induction.[8] Therefore, the increased response to thyroid hormones seems to be specific to TSH, rather than a general pituitary adaptation in fasting and diabetes mellitus.

On the other hand, experimental evidence supports the hypothesis that hypothalamic changes might be the primary cause of impaired TSH secretion accompanying fasting and diabetes mellitus. Depending on the duration and severity of the metabolic disorder, it has been shown that decreased TSH-releasing hormone production and release in the portal circula-

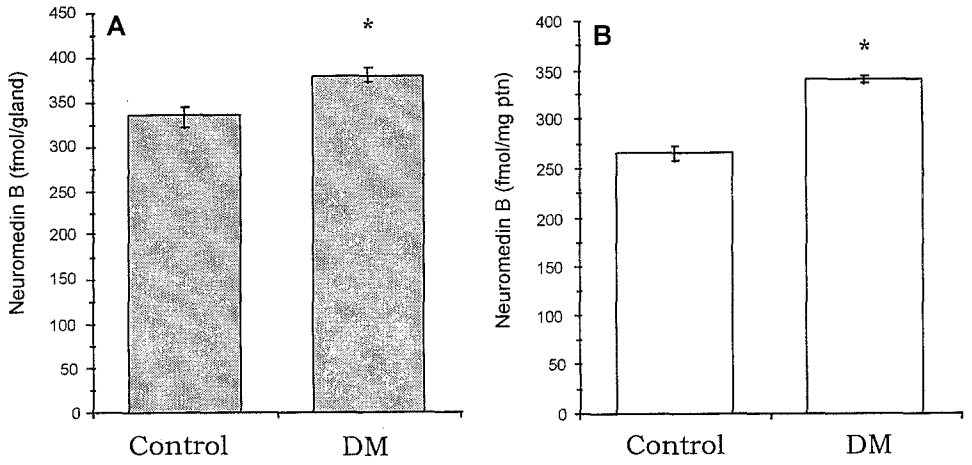

Fig 2. Anterior pituitary neuromedin B content expressed as fmol/gland (A) or fmol/mg protein (B) in diabetic rats (DM) after 20 days of streptozotocin injection. *$P < .05$.

tion[7,22,24] increased somatostatin release.[25,26] This altered hypothalamic input might be responsible for the decreased TSH synthesis, as evidenced by the previously reported reduction in β-TSH and β-TSH mRNA.[7,11] There are no data available concerning a possible modulation of neuromedin B by neurohormones. It was reported that TSH-releasing hormone administered long-term induced an increase in rat pituitary neuromedin B content, which is probably due to the associated hyperthyroidism.[14]

Hypercortisolemia due to the stress associated with the illness might also be involved in the decreased TSH secretion of diabetes mellitus and fasting. However, the effect of the adrenal cortex on pituitary neuromedin B is not clear, since both adrenalectomy and dexamethasone administration were shown to increase the content of the peptide, whereas only the first manipulation also increased neuromedin B mRNA content in the pituitary gland.[15]

In diabetic rats, increased pituitary neuromedin B may also suggest a direct effect of insulin on the peptide production, a hypothesis that needs further experimental support.

The temporal association of the increase in neuromedin B with the decrease in serum TSH in prolonged fasting and with the tendency for low serum TSH in diabetes mellitus might have functional implications. The pituitary is the tissue in the rat that shows the highest neuromedin B concentration.[14-16] It is a locally synthesized peptide, since its mRNA is abundant in the pituitary gland.[15] It has been shown that the synthetic peptide is able to inhibit TSH secretion directly at the pituitary level and at physiological concentrations.[17,18,27] Neuromedin B exerts its effects probably by acting through its own receptors, although they are not as abundant in the gland as receptors for gastrin-releasing peptide,[28] another peptide of the bombesin family that has been reported to inhibit in vitro TSH release in pharmacological doses.[29,30] The role of endogenous neuromedin B was clearly evidenced by the increase in TSH release from isolated pituitaries incubated with an antiserum against neuromedin B. Altogether, the data suggest a local regulatory role as an autocrine factor that inhibits TSH secretion. The positive modulation by thyroid hormones[14,15] and the marked effect of immunoneutralization in isolated hyperthyroid glands[18] also suggest a role for neuromedin B in the mechanism by which thyroid hormones suppress TSH.

In this study, we cannot distinguish whether the decreased or inappropriately normal TSH secretion in the face of low serum thyroid hormones and the increased neuromedin B pituitary concentration are related to each other from the pathophysiological point of view, or are merely epiphenomena. However, we can argue that the increased concentration of an inhibitory locally acting peptide might play a role in the decreased TSH secretion associated with fasting and diabetes mellitus.

A reduction in thyroid function of diabetic and fasting rats may be seen as an adaptative mechanism to a decrease in energy expenditure and protein catabolism. Bombesin-like peptides have been implicated in the central control of glucose metabolism.[31] Injection of neuromedin B into the fourth ventricle of rats induced an increase in blood glucose levels, although it is less potent than bombesin. On the other hand, neuromedin B has an insulin release–stimulating action, although it is more pronounced at pharmacological doses.[32,33] Furthermore, there was one report of decreased neuromedin B mRNA in the median preoptic area of fasting rats.[34] Therefore, although the

Table 2. Serum Concentration of Glucose, T_4, T_3, and TSH and Protein Concentration in the Anterior Pituitary of Diabetic Rats 20 Days After Streptozotocin Injection

Group	No.	Initial BW (g)	Initial BW loss (g)	Glucose (mg/dL)	Protein (mg/pituitary)	T_4 (μg/dL)	T_3 (ng/dL)	TSH (ng/mL)
C	5	228 ± 7.7	—	177 ± 10.5	1.23 ± 0.04	4.14 ± 0.3	20.0 ± 1.4	1.21 ± 0.2
DM	6	227 ± 5.0	25	>350	1.11 ± 0.03*	2.11 ± 0.8	0.9 ± 0.2†	1.02 ± 0.1

Abbreviations: DM, diabetic rats; BW, body weight.

*$P < .05$.

†$P < .001$.

physiological relevance of bombesin-like peptide in the regulation of intermediary metabolism remains to be elucidated, the data from the present study together with previous reports raise the hypothesis that neuromedin B might play a role in the general metabolic adaptation to fasting and diabetes mellitus.

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