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ORIGINAL PAPER

The thyroid hormone mediated effects of insulin on serum leptin levels of diabetic rats

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Abstract In this study, we aimed to evaluate the possible relations of serum leptin and thyroid hormones on insulin treatment of surgically thyroidectomized and streptozotosin induced diabetic group of rats and whether the thyroid hormones control the leptin levels or leptin levels affect the thyroid hormones in DM. The Sprague–Dawley rats were assigned to eight groups: group 1, control; group 2, diabetes (injected intraperitoneally (i.p.) with streptozotocin (stz) 55 mg/kg); group 3, diabetes + insulin (rats were treated with insulin, 7–10 U/kg/day, subcutaneously); group 4, surgically thyroidectomized control; group 5, thyroidectomized + diabetes (3 weeks after the surgical operation, injected i.p. with stz); group 6, thyroidectomized + diabetes + insulin; group 7, thyroidectomized + diabetes + insulin + thyroid hormone (after diabetes induction, rats were treated with insulin and thyroid hormone, levothyroxin sodium (T_4; 2.5 μg/kg); group 8, thyroidectomized + diabetes + insulin + thyroid hormone (T_4; 5 μg/kg). The free and total T_3 and T_4 levels were measured in serum samples by otoanalyzer, and leptin levels were determined by ELISA method. The main finding of our recent study is that the decreased levels of serum leptin during the diabetes, hypothyroidism, and hypothyroidism with diabetes can be regulated in different percentages with the treatment of insulin and various doses of thyroid hormone. The observations in our study suggest the idea that during diabetic hypothyroidism, without thyroid hormone treatment, insulin is not sufficient to balance the metabolic pathways so mediated effects of insulin in leptin regulation via thyroid hormones are an increased possibility.

Keywords Leptin · Diabetes mellitus · Insulin · Thyroid hormone

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia resulting from the impaired function in carbohydrate, lipid, and protein metabolism that leads to long-term complications [1]. The incidence of DM in industrialized nations has increased dramatically over the past two decades and continues to increase. Due to the fact that most patients with type 2 diabetes are also overweight or obese, a putative relationship between leptin and obesity contributing to the pathophysiology of diabetes has been proposed. A potential contribution of leptin is supported by findings showing leptin to have a direct effect on insulin activity and regulating total-body sensitivity to insulin and triglyceride levels in lipodystrophic syndromes [2].

Leptin is an active protein specifically secreted by human and mammalian adipose tissue, which has a feedback regulation on its receptors in the hypothalamus where

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the interaction with its receptor molecule leads to suppression of food intake through the release of corticotropin-releasing hormone and suppression of neuropeptide Y. It has a 16 kD protein consisting of 167 amino acids, which is the product of ob gene, was identified from genetic obesity syndromes, and regulated by neuroendocrine system [3, 4].

It had been thought for a long time that thyroid hormones were the only ones to regulate energy production within mitochondria. However, recent findings show that other hormones (steroids, leptin, and insulin) can also regulate the efficiency of mitochondrial adenosine triphosphate production. Leptin could also be a thermogenic hormone, especially in situations of calorie restriction [5]. As thyroid hormones are major regulators of energy homeostasis, it is possible that leptin and thyroid hormone exert their actions on thermogenesis and energy metabolism via the same common effector pathways. Leptin influences feedback regulation of the hypothalamic TRH-secreting neurons by thyroid hormone. Low serum levels of thyroid hormones reflect a dysfunction of the hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-adrenal (HPA) axis [6]. The absence of leptin or the long signaling form of the leptin receptor (LRb) in *ob/ob* and *db/db* mice, respectively, results in a phenotype of obesity secondary to hyperphagia and decreased metabolic rate (due at least in part to hypothyroidism and decreased sympathetic nervous system tone) [7].

Recently, many researches showed that leptin also plays a role in sympathetic nerve activation, insulin metabolism, haematopoiesis, and angiogenesis besides in regulating ingestion and energy metabolism balance [8].

The purpose of the recent study was to determine the effects of insulin and T_4 or their combination in diabetic, thyroidectomized, or diabetic-thyroidectomized rats and possible relations with serum leptin concentrations.

Material and methods

The study protocol was reviewed and approved by the Animal Care Committee and Surgical Research Center of Gazi University Faculty of Medicine (GUDAM). Guiding principles for experimental procedures found in the Declaration of Helsinki of the World Medical Association regarding animal experimentation were followed in the study. In this study, male (in this type of research models male animals preferred in order to avoid the hormonal challenges) Sprague–Dawley rats, weighting 200–250 g that were about middle age, were maintained with 12 h of light and dark cycle, controlled humidity, temperature, and free access to standard diet and tap water for 7 days prior to experiment and during the experiments. Body weights were controlled for the standardization within the group and between the groups before the experiments, every week during the experiments, and the last measurement was done prior to sacrifice. Animals were killed by intraperitoneal Rompun® (xylazine hydrochlorur) and Ketalar® (ketamine hydrochlorur) anesthesia.

Induction of experimental DM; after an overnight fasting, rats were induced by intraperitoneal administration of streptozotocin (stz) (a total dose of 55 mg/kg). Streptozotocin can primarily destroy the β cells of pancreatic tissue, prevent proinsulin synthesis, and cause DNA chain breaks after decreasing the NAD levels by cytoxic effects [9, 10]. Streptozotocin (Sigma; S-0130) was freshly dissolved in saline and maintained on ice prior to use. Three days after the administration, rats were fasted for overnight and blood glucose levels were determined. The animals that had basal glycemia levels of 400 mg/dl were used in the experiment. Animals had free access to food and water after the stz injection.

Rats were assigned to eight groups: group 1, control; group 2, diabetes; group 3, diabetes + insulin (after diabetes induction, rats were treated with insulin for 5 weeks (7–10 U/kg/day, Insulatard-HM® Penfill®, Novo Nordisk, 100 IU/ml NPH, subcutaneously (s.c.) single injection per day), group 4, surgically thyroidectomized control; group 5, thyroidectomized + diabetes (diabetes was done 3 weeks after the surgical operation at hypothyroid state); group 6, thyroidectomized + diabetes + insulin (after diabetes inductions, rats were treated with insulin for 5 weeks (7–10 U/kg/day, s.c. single injection per day)); group 7, thyroidectomized + diabetes + insulin + thyroid hormone (after diabetes induction, rats were treated both with insulin (7–10 U/kg/day, s.c. single injection per day) and thyroid hormone, levothyroxin sodium, 2.5 μg/kg, Tefor® (Organon for 5 weeks); group 8, thyroidectomized + diabetes + insulin + thyroid hormone (after diabetes induction, rats were treated both with insulin (7–10 U/kg/day, s.c. single injection per day) and thyroid hormone, levothyroxin sodium, 5 μg/kg for 5 weeks). We used two different doses, one was the least and the other was the highest, so as to decide the critical dose.

Blood samples were collected from the tail vein during the experiments and with cardiac puncture at the time of sacrifice. Blood glucose concentrations were immediately determined by glucose oxidase enzymatic assay with an Ames Glucometer (Miles Laboratories Inc., Elkhart, IN, USA). The free T_3 (FT_3), free T_4 (FT_4), total T_3 (T_3), and T_4 (T_4) concentrations were measured in serum samples by otoanalyzer which were a competitive enzyme immunoassay (TOSOH AIA-21, TOSOH Bioscience, N.V., Tessenderlo, Belgium). Serum leptin levels were determined by ELISA method (TiterZyme® EIA, rat Leptin, Enzyme Immunometric Assay Kit, Catalog No. 900-015). Blood samples were centrifuged for 20 min at 2000*g* and

serum samples were stored at −70°C for thyroid hormone and leptin analyses that were done within the 4 months. HbA1c levels were analyzed in the day of experiments by autoanalyzer (Olympus AU 400 analyzer, Olympus Diagnostica GmbH, Clarecastle, Ireland) depending on the turbidimetric inhibition of hemolized blood samples.

Statistical analysis

Statistical analysis was performed using statistical software SPSS for Windows, version 8.0 (SPSS Inc., USA). The P values <0.05 were considered statistically significant for all analyses. All values presented in tables were expressed as mean ± SD. The significance of the difference between the mean of test and control groups was established by Mann–Whitney U-test. Pearson correlation test was used to determine the relationships between continuous variables.

Results

After streptozotocin administration, rats demonstrated polyphagia, polydipsia, polyuria, and stable hyperglycemia 5 weeks as determined by measuring blood glucose levels for every 3 days. Body weight measurements revealed a significant difference in all groups compared to group 2 and also groups 2 and 4 revealed a significant difference compared to groups 1 and 5 at the time of sacrifice ($P < 0.05$). Furthermore, blood glucose determination showed a significant hyperglycemia in groups 2 and 5 compared to group 1 ($P < 0.05$). HbA1c levels were significantly increased in groups 2 and 5 compared to group 1 ($P < 0.05$). Table 1 summarizes the mean changes in body weight, blood glucose concentrations, and HbA1c levels of all groups.

Serum FT_3 (free T_3), FT_4 (free T_4), T_3 (total T_3), and T_4 (total T_4) levels were significantly decreased ($P < 0.05$) in group 2 after 5 weeks of diabetes and serum FT_3 levels were normalized in group 3 depending on insulin treatment compared to control, group 1. In group 5, FT_4 and T_4 levels were significantly decreased after the induction of diabetes ($P < 0.05$) compared to group 4. In groups 7 and 8, both levels of thyroid hormones were significantly increased after the treatment ($P < 0.05$). Table 2 summarizes the mean changes of serum FT_3, FT_4, T_4, and T_3 levels in all groups. The highest concentration of T_3 measurable is approximately 8 ng/ml and the lowest is 0.15 ng/m, recovery was about 91.8–96.8%. The intra-assay precision coefficient of variation was 5%, inter-assay was 4.6% and for T_4, it was 8.7 and 6.7%, respectively. The intra-assay precision coefficient of variation for FT_4 was 8.9%, inter-assay was 9.6%, and for FT_3 was 6.1 and 4.2%, respectively. The results shown in Fig. 1 indicate the serum leptin levels of all groups. The serum leptin levels of all groups except group 8 were significantly decreased ($P < 0.05$) when compared to control, group 1 (3325.9 ± 503.6 pg/ml). In group 2, leptin levels (193.6 ± 94.2 pg/ml) were significantly higher than group 5 (104.3 ± 5.9 pg/ml). In addition, in groups 3, 6, 7, and 8, serum leptin levels were significantly increased when compared to groups 2 and 5.

In groups 4 (112.4 ± 7.3 pg/ml) and 5, serum leptin levels were significantly lower than group 2 and in group 6 levels were significantly increased compared to group 5. In group 8, serum leptin levels (3141.63 ± 143.55 pg/ml)

Table 1 Changes in the body weight, blood glucose concentration, and HbA1c of all groups (values represent mean ± SD)

Groups		Body weight (g)	Blood glucose concentration (mg/dl)	HbA1c (%)
Group 1 ($n = 10$)	Control	235.0 ± 35.3	109.5 ± 4.6	4.82 ± 0.30
Group 2 ($n = 9$)	Diabetes (DM)	159.0 ± 32.2[a,b]	424.0 ± 22.8[a]	12.11 ± 0.60[a,b]
Group 3 ($n = 6$)	DM + insulin (I)	232.1 ± 48.5[b,c]	108.0 ± 18.2[b,c]	4.24 ± 0.07[a,b,c]
Group 4 ($n = 9$)	Thyroidectomy (Thy)	285.0 ± 34.7[a,b,c]	115.8 ± 20.1[b,c]	4.64 ± 0.20[b,c]
Group 5 ($n = 8$)	Thy + DM	235.0 ± 22.7[c]	487.4 ± 35.7[a]	6.01 ± 0.80[a,c]
Group 6 ($n = 9$)	Thy + DM + I	239.5 ± 32.9[c]	109.2 ± 9.6[b,c]	5.38 ± 0.80[a,c]
Group 7 ($n = 4$)	Thy + DM + I + Thyroid hormone (T_4, 2.5 μg/kg)	225.0 ± 5.7[c]	92.7 ± 4.0[b,c]	4.76 ± 0.15[b,c]
Group 8 ($n = 6$)	Thy + DM + I + Thyroid hormone (T_4, 5 μg/kg)	232.3 ± 2.6[c]	92.7 ± 3.1[b,c]	4.92 ± 0.13[b,c]

n = number of rats

[a] Significant compared to group 1, control ($P < 0.05$)

[b] Significant compared to group 5, thyroidectomized and diabetes ($P < 0.05$)

[c] Significant compared to group 2, diabetes ($P < 0.05$)

Table 2 Serum thyroid hormone levels of all groups (values represent mean ± SD)

Groups	Expected reference interval for kit	FT_3 (pg/ml) 2.1–3.8	FT_4 (pmol/l) 9.67–19.86	T_3 (ng/ml) 0.78–1.59	T_4 (nmol/l) 51.6–141.9
Group 1 ($n = 10$)	Control	$3.87 \pm 0.63^{a,b}$	$19.84 \pm 2.15^{a,b}$	$0.64 \pm 0.02^{a,b}$	$40.11 \pm 3.30^{a,b}$
Group 2 ($n = 10$)	Diabetes (DM)	1.39 ± 0.35^{c}	7.54 ± 3.17^{c}	0.28 ± 0.07^{c}	$19.47 \pm 7.23^{b,c}$
Group 3 ($n = 7$)	DM + insulin (I)	$4.33 \pm 0.77^{a,b}$	$28.73 \pm 4.15^{a,b,c}$	$0.41 \pm 0.11^{a,b,c}$	$35.38 \pm 4.06^{a,b,c}$
Group 4 ($n = 10$)	Thyroidectomy (Thy)	1.46 ± 0.08^{c}	$9.14 \pm 2.06^{b,c}$	0.29 ± 0.09^{c}	$15.54 \pm 4.15^{b,c}$
Group 5 ($n = 8$)	Thy + DM	1.39 ± 0.26^{c}	$4.20 \pm 2.31^{a,c}$	0.28 ± 0.05^{c}	$6.54 \pm 0.01^{a,c}$
Group 6 ($n = 9$)	Thy + DM + I	$2.63 \pm 0.98^{a,b,c}$	7.56 ± 2.12^{c}	0.26 ± 0.05^{c}	$20.06 \pm 5.24^{b,c}$
Group 7 ($n = 4$)	Thy + DM + I + thyroid hormone (T_4, 2.5 μg/kg)	$5.10 \pm 0.83^{a,b,c}$	$26.52 \pm 3.00^{a,b,c}$	$0.46 \pm 0.02^{a,b,c}$	$46.27 \pm 6.81^{a,b}$
Group 8 ($n = 6$)	Thy + DM + I + thyroid hormone (T_4, 5 μg/kg)	$7.05 \pm 1.95^{a,b,c}$	$21.07 \pm 9.48^{a,b}$	$0.64 \pm 0.12^{a,b}$	$41.78 \pm 15.80^{a,b}$

n = number of rats

[a] Significant compared to group 2, diabetes ($P < 0.05$)

[b] Significant compared to group 5, thyroidectomized and diabetes ($P < 0.05$)

[c] Significant compared to group 1, control ($P < 0.05$)

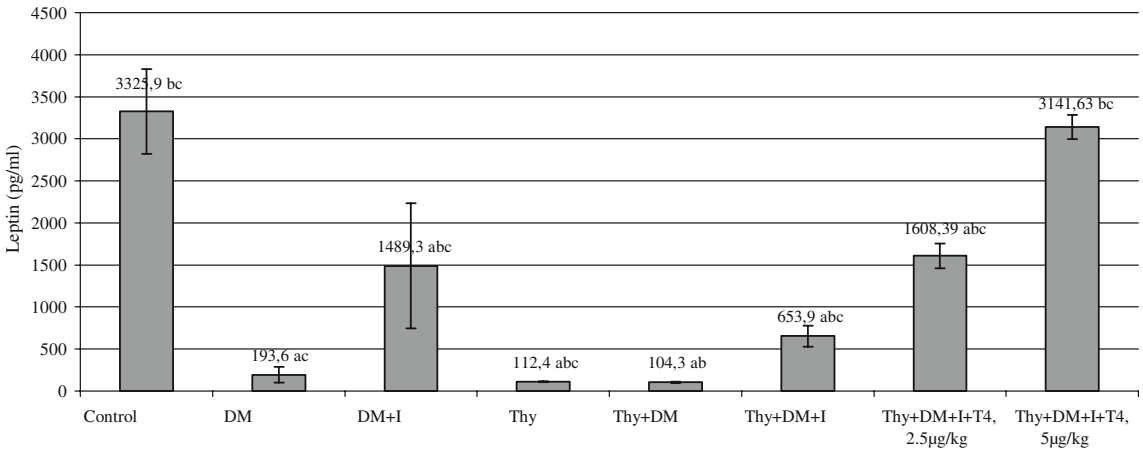

Fig. 1 Serum leptin concentrations of all groups. Data are presented as mean ± SD. [a]Significant compared to group 1, control ($P < 0.05$); [b]significant compared to group 2, diabetes ($P < 0.05$); [c]significant compared to group 5, thyroidectomized and diabetes ($P < 0.05$)

were significantly increased when compared to groups 2 and 5. Negative correlations were observed between leptin and body weights in groups 2 and 3 ($r = -0.953$, $P = 0.001$ and $r = -0.821$, $P = 0.023$, respectively). Also, in group 8, negative correlation was observed between leptin and HbA1c levels ($r = -0.829$, $P = 0.042$).

Discussion

The main finding of our recent study is the decreased serum leptin levels during the diabetes, hypothyroidism, and hypothyroidism with diabetes, which can be regulated in different percentages with the treatment of insulin and various doses of thyroid hormone. These results indicate that thyroid hormone may play an important role in the appropriate secretion of leptin in rats.

Leptin has been known to have a wide spectrum of activity regulating both central endocrinological and physiological processes and peripheral tissue activity from bone growth to regional sympathetic nerve activation.

A number of epidemiological studies have suggested that, independent of body composition, leptin concentration can be affected by both insulin and cortisol which are potent and possibly physiological regulators of leptin expression in human adipose tissue [11, 12]. Müller et al. [13] suggested that leptin could affect the several important metabolic effects of insulin including stimulation of glucose transport, glycogen synthase activity, lipogenesis, and protein synthesis as well as inhibition of iso-proterenol-

induced lipolysis and PKA activation in isolated rat adipocytes. Leptin seemed to exert an insulin-like effect on glucose transport and glycogen synthesis in mouse C_2C_{12} myotubes [14] and modulate the insulin activities in cultured liver cells [15]. In our study, we also observed increased leptin levels in insulin treated diabetic group compared to diabetes group, but this alteration was not as effective as in hypothyroid-diabetic insulin treated group when compared to hypothyroid-diabetic group (Fig. 1, groups (2–3) and (4–5)). We may suggest that the insulin treatment causes dose-related increases in leptin levels in the presence of thyroid hormones. In their study, Fain and Bahoutha [16] also concluded that T_3 could either inhibit or stimulate the net leptin mRNA content of white adipose tissue with the absence or the presence of insulin.

Low leptin levels, caused by deficiency or destruction of adipocytes, are associated with abnormalities such as hypertriglyceridemia and severe insulin resistance which are usually accompanied by DM [17]. In our diabetes group, decreased body weight with decreased leptin levels also in accordance with that idea, however, we did not observe increased leptin levels in thyroidectomized group although the body weights were significantly increased. Perros et al. also suggested that thyroid function should be screened annually in diabetic patients to detect asymptomatic thyroid dysfunction, which is increased in frequency in a diabetic population [18], and increased levels of thyroid hormones were showed after the insulin treatment [19]. Our stz-induced diabetic group data support this knowledge, showing a decrease for all serum thyroid hormone parameters in diabetes groups and an increase after insulin treatment. In addition to those, also in this study, low leptin levels can be explained with inadequate thyroid hormones synthesis in surgically hypothyroid and hypothyroid with diabetes group. In these animals, serum leptin levels were significantly lower in both groups compared to diabetes group. Hormonal dysregulation in DM include not only insulin but also thyroid hormones, which may have metabolic roles on the dysregulation of leptin in diabetes. In various studies, leptin levels showed differences from one to another. Karakoc et al. [20] suggested increased serum leptin concentrations in hypothyroidism while Syed et al. [21] found decreased levels in hypothyroid rats. We also found significantly decreased leptin levels in thyroidectomized group ($P < 0.05$). It is not elucidated that whether the thyroid hormones control the leptin levels or leptin levels affect the thyroid hormones in DM.

Aside from being the food intake inhibitor and the energy control factor, leptin takes part in controlling the pituitary hormones. Leptin promotes the secretion of growth hormone, prolactin, thyroid stimulating hormone-beta (TSH-β), follicule stimulating hormone β/luteinizian hormone β, and inhibits the secretion of adrenocorticotropic hormone, which are the major changes of pituitary hormones [22]. Also, leptin regulates thyrotropin-releasing hormone (TRH) production in the parvocellular paraventricular nuclei to participate in the activation of the HPT axis [6], apparently by a combination of direct and indirect mechanisms.

The metabolic pathway of hypothyroid state during diabetes is not clear. The cause of lack in thyroid hormones and insulin in diabetes may influence leptin secretion, so altered leptin levels in diabetes may be regulated not only with insulin but also thyroid hormones.

The observations in our study suggest the idea that during diabetic hypothyroidism, insulin is not sufficient to balance the metabolic pathways, so mediated effects of insulin in leptin regulation via thyroid hormones are an increased possibility.

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