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Development of a radioimmunoassay for the measurement of human leptin in serum

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Abstract

BACKGROUND: Leptin is a 16 kDa polypeptide hormone encoded by the obese gene (*ob*) and secreted by adipose tissue. This hormone plays a major role in energy homeostasis and regulation of food intake and body weight. It also affects the metabolic, neuroendocrine and reproductive systems.
MATERIAL AND METHODS: Labelling of recombinant human leptin with ^{125}I was best performed by the Chloramine-T method. New Zealand white rabbits were immunised with recombinant human leptin, cross-reaction of obtained antisera was analyzed with 10 different antigens. The separation of bound and free fractions was performed using the second antibody — PEG method.
RESULTS: The obtained tracer had specific activities of 2.8–3.3 kBq/μg and had a stability of 5 weeks. A highly specific polyclonal antibody was obtained without measurable cross-reaction against the analysed antigens. Concentrations of human leptin were measured by a single overnight incubation assay with a sensitivity of 0.5 ng/ml and a measuring range of 0.5–100 ng/ml. The intra-assay and inter-assay coefficient of variation was under 6% and 8%, respectively. Recovery ranged from 88% to 106%.
CONCLUSIONS: Serum human leptin concentrations can be accurately and precisely measured by this new radioimmunoassay. Preliminary results obtained from the measurement of serum leptin in lean, overweight and obese patients are presented. Serum leptin concentrations correlated with body mass index and were significantly higher in women than in men, except for obese patients.
Keywords: **radioimmunoassay, leptin, ^{125}I, obesity, BMI**

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Introduction

Zhang and co-workers reported in 1994 the discovery of leptin, a hormone secreted primarily by adipose tissue [1]. Leptin is encoded by the obese (*ob*) gene, which has been characterised in a number of animal species as well as in humans. After the intracellular processing of a 167 amino acid pro-hormone to cleave a 21 amino acid signal peptide segment. The mature form of leptin is secreted into the bloodstream where it circulates as a 146 amino acid (16 kDa) protein [1]. Crystal structure and NMR studies have characterised leptin as a four-helix bundle containing one disulphide bond (Cys_{96}– Cys_{146}) that is essential both to its structure and to its function [2, 3].

Leptin receptors, similar to those of the class I cytokine receptor super-family, have been identified in the central nervous system as well as in several peripheral tissue locations [4]. In the hypothalamus, leptin has been reported to suppress neuronal levels of neuropeptide-Y and agouti-related peptide, while stimulating the production of proopiomelanocortin [5]. These actions, together with other less well characterised signalling pathways bring about a suppression of feeding behaviour as well as changes in energy expenditure and body weight. It has been shown that leptin levels are highly correlated with body fat and body mass index (BMI) [6]. *In vitro* studies indicate that insulin and glucocorticoids work directly on adipose tissue to upregulate leptin mRNA levels and rates of leptin secretion, while catecholamines chronically decrease leptin expression and secretion [7].

Leptin also acts at multiple levels of the reproductive axis, eliciting different effects on reproduction depending on its blood concentrations. The saturable system of leptin transport through the blood-brain barrier prevents high levels of leptin reaching hypothalamic receptors, where leptin exerts stimulatory actions on gonadotropin-releasing hormone and in turn gonadotropin secretion. In contrast, excess levels of leptin have the potential to act on peripheral leptin receptors and exert inhibitory actions on testicular and ovarian steroidogenesis [8].

Leptin and the related peptides, which participate in appetite and feeding behaviour, seem to function together to regulate cardiovascular system and sympathetic nerve activity, and play a key role in the association between obesity and hypertension [9].

We aimed to develop a specific method to measure leptin in human sera. This radioimmunoassay would help clinicians in the endocrinological research and treatment follow-up of obesity and related disorders.

Material and methods

Amounts of 10 μg recombinant human Leptin (rhLeptin) (SIGMA-ALDRICH) were labelled with 37–56 MBq ^{125}I (Institute of Isotopes, Budapest) by two methods, using Chloramine-T (SIGMA) or iodo-gen (PIERCE) as oxidising agents. The efficiency of labelling was determined on thin layer (TLC) chromatography (MERCK) developed by acetone (REANAL, Hungary). The purification of the tracer was performed by gel filtration on a Sephadex G-50 column (PHARMACIA), fractions of 0.5 ml were collected. Labelling efficiency and immunological stability were the main conditions for selecting the best performing labelling method. The purified tracer was diluted to a concentration of 25000 cmp/100 μl and stored at 4°C. Dilution buffer: 0.05M phosphate (REANAL) buffer pH 7.4 containing 1% BSA (SIGMA), 0.05% Triton X-100 (REANAL), 0.1% NaN_3 (REANAL) and 0,01 mg/ml Congo-Red colouring agent (MERCK).

Two New Zealand white rabbits were immunised intradermally on multiple sites, each with 200 μg rhLeptin mixed with complete Freund's adjuvant (DIFCO LABORATORIES). The immunisation was repeated three times at three week intervals with 100 μg, and then the rabbits were bled. The specificity of antiserum was examined with ten antigens (Tab. 1).

Standard solutions were prepared with rhLeptin diluted in sterile horse serum (University of Veterinary Medicine, Kaposvár, Hungary), then samples of 0.5 ml were lyophilised and stored at 4°C. For the radioimmunoassay, quantities of 100 μl of standards, unknown samples, antiserum and tracer were used.

The antigen — antibody reaction kinetics were analyzed between 3 and 48 hours at two temperatures: 4°C and room temperature. The effects of shaking and pre-incubation with the antibody were also tested.

The separation of free and bound fractions was performed using a 1 ml mixture of anti-rabbit IgG (second antibody raised in sheep, University of Veterinary Medicine, Budapest) and 8% PEG (REANAL). After 20 minutes incubation at room temperature, the mixture was centrifuged at 2000 g for 20 minutes and the supernatant decanted. The radioactivity in the pellets was counted to determine bound radioactivity.

Table 1. Specificity of anti-Leptin polyclonal antibody

Antigen	Concentration	Cross-reaction
Glucagon (Serono)	800 pg/ml	nd
IGF-1 (Schering-CIS)	1050 ng/ml	nd
Insulin (Institute of Isotopes)	400 mUI/l	0.03%
C-Peptide (Institute of Isotopes)	30 ng/ml	nd
TNF-α (Calbiochem)	200 ng/ml	nd
IL-1β (Calbiochem)	40 μg/ml	nd
HPL (Aalto Bio Reagents)	20 μg/ml	nd
FSH (Pharmacia)	150 mUI/ml	nd
PRL (Calbiochem)	400 ng/ml	nd
HCG (in-house)	100 μg/ml	nd

nd — not decectable

For assessing the imprecision of our assay, we determined the intra-assay and inter-assay coefficient of variation from 10 replicates of 3 and 4 serum samples, respectively. A dilution test was performed using 4 serum samples and the recovery percentages were calculated.

Leptin concentrations in serum samples of 35 lean (BMI 17–26 kg/m^2), 45 overweight (BMI 27–35 kg/m^2) and 44 obese (BMI 36–68 kg/m^2) fasting adult patients (50 men and 74 women, Szent Imre Hospital, Budapest) were measured with our RIA. The results were compared by means of unpaired, two-tailed t-tests and we also established the correlation between leptin concentration and BMI for men and women, separately.

Results

Both labelling methods proved to be satisfactory regarding labelling efficiency. Values of 75–90% were obtained, as calculated from TLC strips (Fig. 1). However, leptin labelled by the iodo-gen method did not show a good antibody-binding capacity, only 30–35% maximum binding values were obtained in contrast with leptin labelled by the Chloramine-T method, where these values moved between 45 and 50%. For this reason, we chose Chloramine-T labelling method for obtaining our tracer. Fractions of high radiochemical purity (> 98%) were obtained after purification by gel filtration (Fig. 2), as reassessed by TLC. The specific activity of the tracer was between 2.8 and 3.3 kBq/μg (mean 3 kBq/μg, n = 10). The tracer was stable for at least 5 weeks.

Two polyclonal antibodies were obtained from the immunised rabbits, with titers of 6000 × and 5000 ×, respectively. The first one was selected and its specificity analysed with 10 different antigens, not showing any significant cross-reaction (Tab. 1).

To reach the kinetic equilibrium 18–24 hours are required at 4°C. Shaking the mixture can not reduce the reaction time. The sensitivity of the assay can be increased by incubating at room temperature and/or pre-incubating overnight with the antiserum. However, this "improvement" would bring a displacement of the measuring range towards the lower concentrations, then samples with a leptin concentration between 50 and 100 ng/ml (as in the case of most overweight and obese patients, see later) could not be accurately measured. For this reason, we chose a one-step assay with an overnight incubation at 4°C. Under these

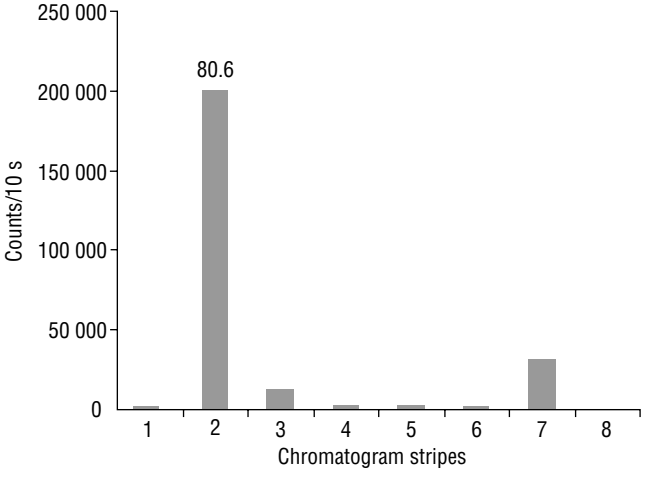

Figure 1. Thin layer chromatogram of labelling mixture, determination of labelling efficiency.

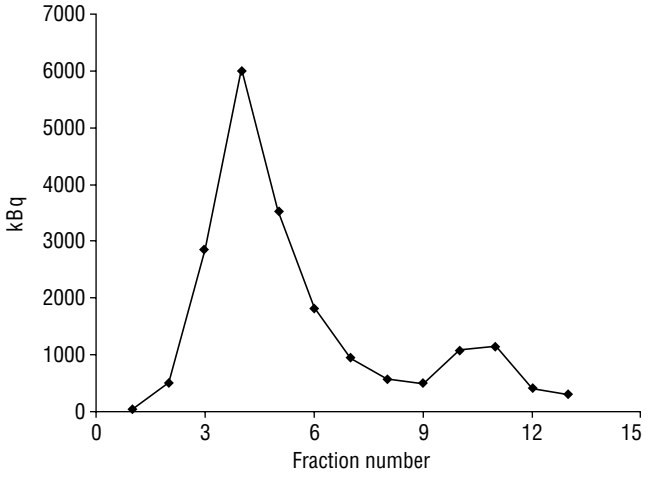

Figure 2. Purification of ^{125}I-leptin. Elution curve from Sephadex G–50 column.

Table 2. Intraassay and Interassay imprecision

INTRAASSAY				INTERASSAY				
Sample	A	B	C	Sample:	D	E	F	G
	4.30	18.52	45.97		2.50	4.79	10.01	51.43
	4.10	17.95	46.16		2.70	5.14	10.81	45.78
	4.30	18.28	46.98		2.28	4.41	10.01	49.67
	4.16	15.75	39.60		2.53	5.62	10.18	50.30
	4.37	18.70	43.85		2.36	5.1	10.53	47.38
	4.54	16.76	45.37		2.66	4.98	9.36	47.83
	4.12	18.21	47.30		2.59	5.31	10.73	46.36
	4.50	17.39	45.31		2.44	5.05	9.62	52.02
	4.10	17.06	42.01		2.8	4.84	9.36	44.97
	4.37	18.24	44.28		2.9	5.52	10.64	50.04
Mean	4.9	17.69	44.68	**Mean**	2.58	5.08	10.13	48.58
SD	0.16	0.93	2.37	**SD**	0.19	0.36	0.55	2.45
CV%	**3.79**	**5.27**	**5.31**	**CV%**	**7.53**	**7.02**	**5.43**	**5.04**

Table 3. Dilution test

Dilution	Measured (ng/ml)	Expected (ng/ml)	Recovery	Measured (ng/ml)	Expected (ng/ml)	Recovery
Sample:			H			I
1 ×	49.23			34.27		
2 ×	21.9	24.62	**89%**	15.1	17.14	**88%**
5 ×	9.05	9.85	**92%**	6.27	6.85	**91%**
10 ×	5.22	4.92	**106%**	3.04	3.43	**89%**
Dilution	**Measured (ng/ml)**	**Expected (ng/ml)**	**Recovery**	**Measured (ng/ml)**	**Expected (ng/ml)**	**Recovery**
Sample:			J			K
1 ×	87.4			76.27		
2 ×	42.08	43.70	**96%**	33.88	38.14	**89%**
5 ×	16.63	17.48	**95%**	14.57	15.25	**96%**
10 ×	9.12	8.74	**104%**	8.03	7.63	**105%**

conditions, a wide measuring range (0,5–100 ng/ml) makes it possible to determine leptin concentrations in both lean subjects and obese patients with good sensitivity (0.5 ng/ml).

The calculated intra-assay and inter-assay coefficient of variations were under 6% and 8%, respectively (Tab. 2). The recoveries obtained from the dilution test were between 88% and 106% (Tab. 3).

Mean serum leptin concentrations of lean, overweight and obese patients are shown in Table 4. Serum leptin concentrations were significantly higher in lean women than in lean men ($p < 0.000001$) and in overweight women than in overweight men ($p < 0.0001$), but this difference was not statistically significant in the case of obese men and women ($p = 0.09$). The linear correlation coefficient between BMI and leptin concentration was 0.71 for men (Fig. 3) and 0.52 for women (Fig. 4).

Table 4. Leptin concentrations measured in serum samples of lean (BMI 17–26), overweight (BMI 27–35) and obese (BMI 36–68) patients

BMI (kg/m²)	Men n	Leptin (ng/ml) Mean ± SD	Women N	Leptin (ng/ml) Mean ± SD
17–26	14	**6.63** ± 3.37	21	**17.65** ± 6.16
27–35	18	**20.42** ± 17.46	27	**51.39** ± 24.94
36–68	18	**53.46** ± 39.23	26	**80.36** ± 62.73

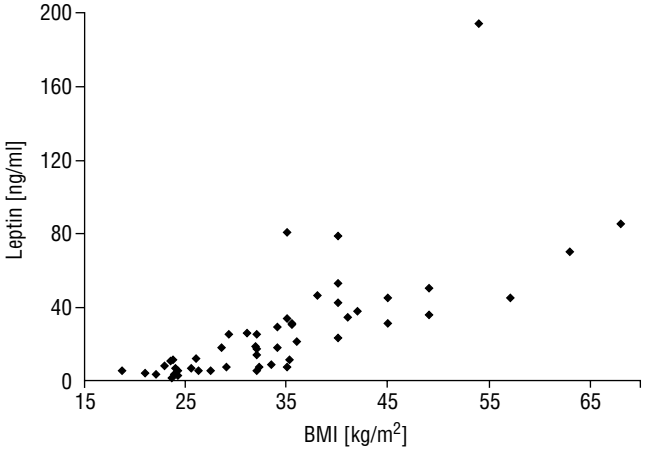

Figure 3. Correlation between BMI and serum Leptin concentrations in men.

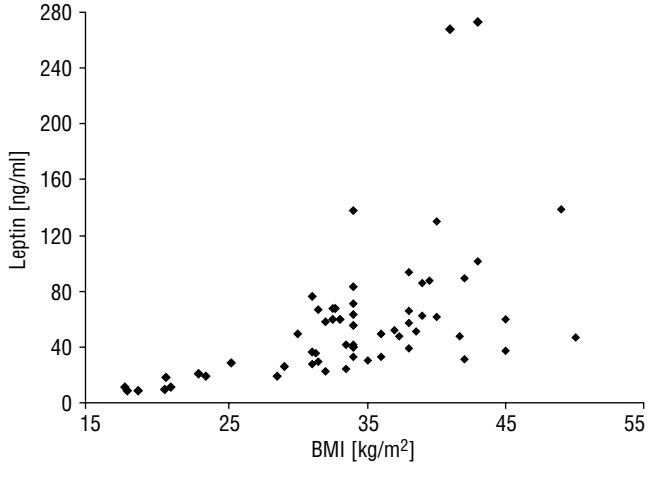

Figure 4. Correlation between BMI and serum Leptin concentrations in women.

Discussion

The radioiodination of human leptin has been reported by the Chloramine T [10] and the Bolton-Hunter method [11]. In both cases, lower specific activities were obtained: 1,1 kBq/μg [11] and 2.3 to 7.2 kBq/μg, respectively. In spite of this, Considine et al [11] report only 30 days of stability. This could be due to the differences in storage; we attempted to optimise the composition of the dilution buffer and the grade of dilution of the tracer in order to achieve the longest possible stability. To our knowledge, iodination with the iodo-gen method has not been reported to date. In our study, the tracer obtained by this method partially lost its antibody-binding capacity, although a similar radioactive incorporation was obtained by the Chloramine-T method without showing the same problem. We suppose that during iodo-gen labelling the iodine not only reacts with the 4 tyrosine amino acids present in the leptin molecule but also (or preferably) with the 2 histidine amino acids, too. One of these histidines is placed just beside the disulphide bridge, which is critical for the structural integrity and stability of the molecule [12]. This bond could be damaged or sterically modified by the presence of the iodine, affecting in this way, the immunological properties of the protein.

The specificity of anti-leptin antibodies has been commonly analysed only with leptins from animal specimens and with diabetes-related hormones: insulin, glucagons, C-peptide and IGF-1 [11]. (Commercially available kits from Linco Research Inc, DRG International Incorporation, Diagnostic Systems Laboratory Incorporation, Immuno Biological Laboratories, Hamburg GmbH). We tested the specificity of our antiserum with a broad spectrum of human antigens, taking into account the important role that leptin plays also in the reproductive system and the fact that leptin has a helical structure similar to that of the long-chain cytokine family. The absence of cross-reaction found is not totally surprising because to date there is no known sequential homology between leptin and other molecules [2, 3].

The sensitivities of other radioimmunoassays reported are similar to ours: 0.2 ng/ml [10], 0.4 ng/ml [11] and 0.5 ng/ml [13]. Improving sensitivity could be desirable when very small concentrations of leptin should be measured, as in the case of cerebrospinal-fluid samples [14]. In our system, this can be achieved by the overnight pre-incubation with the antibody and by incubating the reaction mixture at room temperature, instead of at 4°C. However, our goal was to design a RIA for measuring serum samples of normal, overweight and obese patients, when concentrations are over 3 ng/ml, according to our data. The measuring range is acceptably wide, nonetheless six samples from obese patients (1 man and 5 women, BMI 34–54 kg/m²) had leptin concentrations over 100 ng/ml (two of them over 200 ng/ml) and required dilution.

As expected, serum leptin concentrations were higher in women than in men, however in the case of obese subjects (BMI $>>$ 36 kg/m²) this difference was not statistically significant. This could imply a "convergence effect" that could be explained by the fact that percentages of body fat in obese men and women become more similar than in lean men and women. Considine et al [11] found significant differences in leptin concentrations between men and women when the groups were defined by BMI but not when defined by percentages of body fat. These results seem to confirm the conclusion of others [15], that BMI is a less precise index of

adiposity than the direct measurement of body fat content. However, serum leptin concentration should not be considered as a direct indicator of adiposity because of all the hormonal factors affecting leptin secretion. More studies are necessary to evaluate the complexity of the leptin-system and the real use of leptin measurements for the investigation of obesity and its relationship with other diseases as diabetes and sterility.

Measuring leptin levels with this newly developed assay will be a tool in further investigations about the onset of obesity, its regulatory mechanisms and in monitoring the effects of treatment protocols.

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