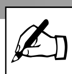

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Technological difficulties in ghrelin and obestatin assays

Trudności technologiczne w oznaczaniu stężenia greliny i obesatyny

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Abstract

In recent years we have performed more than 1,000 radioimmunoassays of ghrelin and obestatin. In these assays, we have encountered several technological obstacles. Another difficulty was the enormous discrepancy of plasma ghrelin results published by different authors. The aim of this article is to comment on these problems. Not all peptides of the hypothalamus and intestines are present in blood circulation. Several neuropeptides do not cross the blood-brain barrier, and several gastrointestinal peptides are present in extremely low concentrations in the blood. That requires time-consuming and laborious extraction. In these procedures, considerable amounts of peptides may be lost. In addition, these peptides are very unstable and prone to enzymatic degradation. This makes it mandatory to add enzymatic inhibitors to plasma samples. The peptides are also unstable in elevated temperatures, hence the assays should be performed in air-conditioned laboratories and the kits should be transported in proper low temperature conditions. Peptides may appear in several isoforms of different biological activity, but antibodies routinely used in these assays are polyclonal and do not differentiate between these forms. This complicates clinical evaluation of the results. To date, there are no international standards of ghrelin, obestatin or other active peptides, probably because of their extreme instability. Because of technological difficulties, the results of peptide assays performed in different scientific research institutions vary greatly and cannot be compared to each other. This disadvantage may be partially diminished by including samples of healthy subjects in each assay run to check whether the peptide concentrations of the patients differ significantly from that of control subjects. **(Pol J Endocrinol 2011; 62 (4): 336–339)**

Key words: *ghrelin, obestatin, assays, technological difficulties*

Streszczenie

W trakcie kilkuletnich doświadczeń wykonano ponad 1000 radioimmunologicznych oznaczeń greliny i obestatyny, przy których wykazano liczne trudności techniczne. Inną trudnością była niezwykle wielka rozbieżność wyników greliny w publikacjach różnych autorów. Celem pracy jest omówienie tych problemów.

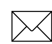
Tylko niektóre peptydy podwzgórza i przewodu pokarmowego są możliwe do oznaczania we krwi krążącej, bowiem część neuropeptydów nie przekracza bariery krew–mózg. Niektóre hormony przewodu pokarmowego występują w niezwykle małych stężeniach. Wymaga to wykonania żmudnych i pracochłonnych ekstraktów, w trakcie których dochodzi do strat części peptydów. Ponadto peptydy są nietrwałe i łatwo ulegają rozkładowi. Są one także wrażliwe na wysokie temperatury, stąd niezbędne jest zachowanie niskich temperatur otoczenia w trakcie wykonywania oznaczeń oraz dodawanie do osocza substancji hamujących aktywność enzymów. Peptydy mogą występować w postaci izoform oraz form aktywnych i nieaktywnych, co utrudnia ocenę wyników badań, bowiem obie formy mogą mieć odmienne działanie biologiczne. Jednak stosowane w zestawach RIA przeciwciała są poliklonalne i nie różnicują tych izoform. Brak międzynarodowych standardów uniemożliwia porównywanie wyników badań różnych ośrodków naukowych. Dlatego w każdej serii oznaczeń powinna być dołączona grupa kontrolna osób zdrowych w celu sprawdzenia, czy wyniki stężeń peptydów osób chorych różnią się statystycznie od uzyskanych u osób zdrowych. **(Endokrynol Pol 2011; 62 (4): 336–339)**

Słowa kluczowe: *grelina, obestatyna, oznaczenia, trudności techniczne*

Introduction

Ghrelin and obestatin are derived from the same precursor (preproghrelin). Ghrelin is a 28-aminoacid peptide discovered by Kojima et al. in 1999 [1], produced mainly in the X/A cells of the stomach and, to a lesser extent, by the intestines, pancreas, hypothalamus and pituitary. Initial studies in rodents [2] followed by studies in humans revealed that ghrelin stimulates GH, ACTH and prolactin release, increases appetite and food uptake [4, 5] by acting on the hypothalamus arcuate nucleus and increasing secretion of neuropeptide Y (NPY) and agouti-related peptide (AgRP). In humans, Cummings studied ghrelin secretion in physiological conditions and presented detailed results of 24-hour ghrelin secretion profile; ghrelin level increased before meals and decreased rapidly after meals [6].

Preproghrelin also encodes another 23-amino acid peptide discovered by Zhang et al. in 2005 [7]. This ghrelin-associated peptide was named obestatin, implying its anti-obesity action. In their study, this peptide decreased appetite, food intake and body mass in



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rodents, thus suggesting its antagonistic effects to the well-known properties of ghrelin. The scientists hoped for a breakthrough in the treatment of obesity, but other authors were unable to confirm these observations as they failed to find alterations in feeding behaviour in either rodents or humans [8–12]. Scientific opinion still differs as to whether obestatin is biologically active or just an inactive accessory peptide fragment originated during ghrelin processing.

Not only ghrelin, but also agouti-related protein (AgRP) and neuropeptide Y (NPY), belong to the appetite-stimulatory peptides. Their main activity is restricted to hypothalamic neurons and ghrelin-producing gastric X/A cells. These peptides do not cross the blood-brain barrier, hence mechanisms and effects of these peptides can be analysed only in experimental studies in rodents or by immunohistochemical and in-situ hybrydisation of the hypothalamus.

In addition, the activity of these peptides may be observed after intraventricular injections of ghrelin or obestatin and studies of how these peptides affect feeding behaviour in experimental animals. AgRP and NPY cannot be measured in circulating blood, meaning that papers on NPY published 20 years ago are no longer valid. Moreover, since then, no further papers on NPY in blood have been published. Among appetite-stimulating peptides, only ghrelin is present in the circulation in quantities that allow its measurement.

In the Department of Endocrinology in recent years we have performed more than 1,000 ghrelin and obestatin radioimmunoassays, and some results of these studies have been published [15–20]. In performing RIA assays, we have come across several technological problems and difficulties which need be taken into consideration to achieve reliable results. These difficulties involve not only ghrelin and obestatin, but also other hypothalamic and gastrointestinal peptides. Until now, only ghrelin could be measured directly in plasma, with other peptides requiring tedious and time-consuming extraction methods to remove interfering substances and to concentrate the peptides. Table I sets out the procedure needed to prepare obestatin or other peptide extracts from plasma.

Plasma must be acidified with an equal amount of 1% trifluoroacetic acid (TFA) and the sample is centrifuged for 10,000 × g (18,000 rpm) for 20 minutes in a cooled ultracentrifuge (Beckman). The SEP-column containing 200 mg of C-18 is activated by 3 ml of 60% acetonitrile in 1% TFA and washed by 10 ml of 1% TFA. The acidified plasma is transferred to the column and the column is washed with 10 ml of 1% TFA and the eluate discarded. The retained obestatin is slowly eluted from the column by the addition of 3 ml of 60% acetonitrile in 1% TFA; this step takes three hours. The eluate is deeply frozen, freeze-dried overnight, and then stored at –70°C until use. Before radioimmunoassay, the samples are dissolved in 0.3 ml of the RIA buffer and are determined in duplicate. The preparation of obestatin extracts requires a laboratory with special equipment i.e.: ultracentrifuge, vacuum centrifuge, centrifuge with cooling system, freeze-dryer and SEP pack columns. To sum up, the procedure of preparing obestatin extracts is difficult, time-consuming and cumbersome. Each step is charged with some loss of isolated peptides, thus the efficiency of extraction varies from 65% to 85%; this could be controlled by adding ^{125}I obestatin to the plasma control sample and measuring its content in the final extract.

Table I. *Peptide extraction procedure from plasma*
Tabela I. *Procedura ekstrakcji peptydów z osocza*

Collect blood into tubes containing EDTA and Trascloan to inhibit plasma proteolityc enzymes
Spin blood samples in cooled centrifuge and collect plasma
Acidify the plasma with concentrated HCl solution
Ultracentrifuge the samples at 10,000 × g for 20 minutes at 4°C and discard the precipitate
To remove plasma proteins, perform liquid chromatography on activated C18 SEP column
Wash the column with 1% trifluoroacetic acid (TFA)
To elute the peptide retained on the column, add 60% acetonitrile in 1% TFA
Concentrate eluate on the Speed Vac centrifuge and freeze-dry overnight
Dissolve the eluate just before assay

Furthermore, the assays procedure requires a proper room temperature in the laboratory, because a temperature higher than 24°C causes rapid peptide degradation and inactivation. Other methods of peptide measurement are even more time-consuming and difficult, such as high pressure liquid chromatography with simultaneous mass spectrophotometry.

The next difficulty to discuss is the marked instability of peptides, both in circulation and in plasma extracts, and the loss of their biological activity in the isolation process. The prolonged storage of ghrelin samples and obestatin extracts leads to peptide degradation. For this reason, the leaflets included RIA and ELISA kits contain recommendations to store the kits for no longer than one month at low temperatures. The manufacturer recommends using RIA kits as soon as possible after delivery, although laboratory staff tend to ignore this advice, and to use the kits right up to the end of the expiration date on the reagents.

Some peptides occur in two different forms: a biologically active form and an inactive form. For instance, ghrelin occurs in an acylated, biologically active form, and in a desacylated inactive form. Antibodies included in

Table II. *Ghrelin concentrations reported by various authors*
Tabela II. *Stężenia greliny podawane przez różnych autorów*

Plasma ghrelin concentration in controls [pg/ml]	Authors [ref no.]
514 ± 63	Otto et al. [21]
124 ± 11	Caminos et al. [22]
109 ± 24	DelParigi [23]
380.5	Bellone et al. [24]
1,870 ± 195	Riis et al. [25]
125 ± 110	Bergmann et al. [26]
264 ± 38	Morpurgo et al. [27]
3,127± 397	Rojdmark et al. [28]
561 ± 32.1	Gimenez-Palop et al. [29]
54.1 ± 35.5	Altinova et al. [30]
50.5 ± 34.8	Altinova et al. [31]
150.84 ± 75.87	Ruchała [18]
686 ± 53.7	Gjedde et al. [32]
275.5 ± 47.9	Kosowicz et al. [16]
985 ± 64.2	Xin et al. [33]
453	Tanaka et al. [34]
85	Malendowicz et al. [35]
2,345	Braclik et al. [36]

RIA kits contain polyclonal antibodies that measure total ghrelin and do not differentiate between the two forms.

Another difficulty is that certain peptide antibodies give cross-reactions with other peptides. Thus it is important to check the specificity of antibodies. Introducing monoclonal antibodies into the next generation of RIA kits will probably greatly enhance the specificity of the assays and allow the achievement of more consistent results from different laboratories.

There are as yet no international standards for biologically active peptides of the hypothalamus or gastrointestinal tract, probably because of the rapid degradation of these peptides. Therefore results obtained in different laboratories vary greatly. Particular laboratories obtain different and probably divergent results even when the RIA kits used are from the same manufacturer.

Table II presents varying results published by different authors. Some of them found a ghrelin concentration below 100 pg/ml [30, 31, 35]; this may result from improper handling of the reagents and inappropriate conditions of transportation, storage and laboratory procedures, that require temperatures below 24°C. In hot countries, air conditioning in laboratories is essential. Publications from some laboratories [25, 28, 36] have reported surprisingly high ghrelin concentrations, above 1,000 pg/ml. This phenomenon is difficult to explain, because the available ghrelin standard curve gives reliable results in the working range from 50 to 1,000 pg/ml and the authors do not explain in these papers how they measured such high results. Even in laboratories of the highest scientific standards, such as Aarhus Hospital in Denmark, plasma ghrelin concentration in controls was 1,870 pg/ml in 2003 [25], whereas in 2008 was 686 pg/ml [32].

At present, it is not possible to exactly define the normal plasma ghrelin and obestatin concentrations. However, most scientific publications report a ghrelin concentration range of 150–600 pg/ml and this was what we found in our study.

In some studies, the results of plasma ghrelin concentration using kits of Linco give results several times higher than results obtained using Phoenix kits [37, 38]. In RIA kit packages, the declared concentration of ghrelin in the control sample given by the manufacturer greatly differs; for example, a control sample may contain 53 or 102 pg/ml of ghrelin; therefore in patient samples the ghrelin concentration could also be two times greater, or half the size. Including the samples of healthy control subjects would partially remove these obstacles encountered in commercial RIA kits and achieve more reliable results to each RIA run. This would allow an estimation of how the results obtained in patients differed statistically from the control group.

An additional cause of laboratory error in ghrelin assays is the requirement to dissolve the ghrelin stock solution 10,000 times to get the highest point of the standard curve. This leads to unavoidable ghrelin loss by absorption on glass and plastic pipette tips.

Conclusions

Only some hypothalamic or intestinal peptides cross the blood-brain barrier and are present in the circulation and thus eligible to assay.

The concentration of several peptides is extremely low. These peptides require laborious extraction procedures and sophisticated laboratory equipment.

Hypothalamic and intestinal peptides are labile and prone to rapid degradation by blood and tissue enzymes. This requires the addition of enzymatic inhibitors and the storage of the reagents at low temperature.

The occurrence of active and inactive forms of peptides and their isoforms may give different results when using polyclonal antibodies contained in commercially available kits, which measure a total of peptide and do not differentiate between these forms.

A lack of international standards of several active peptides (including ghrelin and obestatin) and technological difficulties in the assays hinder direct compari-

sons of results originating from different scientific institutions. In each assay run, a control group of healthy subjects should be included to allow evaluation of how the patient's results differ statistically from controls.

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