Methodological considerations for ghrelin isoforms assay in clinical evaluation in anorexia nervosa

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**A B S T R A C T**

The growing interest concerning the role of metabolic sensors in various eating disorders requires the implementation of a strict methodology to collect, store and process blood samples in clinical studies. In particular, measurement of isoforms of the appetite-stimulating hormone, ghrelin, has been challenging in clinical settings. Indeed the acyl ghrelin (AG) isoform is rapidly degraded into desacyl ghrelin (DAG) by blood esterases, thus optimal conditions for the conservation of AG and accurate determination of AG/DAG ratio should be used. Here, we compared different protease inhibitors (Aprotinin, PHMB, AEBSF) during blood collection, increasing delays (0-180 min) before centrifugation, plasma supplementation with various HCl concentrations, storage durations of frozen plasma (8 and 447 days) and immunoenzyme-assay procedures (one-step versus sequential) in healthy subjects. Optimal conditions were obtained by collecting blood with aprotinin and supplementation of plasma with 0.1 N HCl with subsequent freezing for at least 8 days and using one-step assay. Under such conditions, different patterns of secretion of ghrelin isoforms were characterized in patients with restrictive-type anorexia nervosa (AN-R) before and after nutritional recovery. We illustrate the pulsatile variations of ghrelin isoforms according to the time around a meal and hunger rates in 3 patients with AN-R. This study offers a comprehensive comparison of various conditions using selective and specific immunoassays for both ghrelin isoforms in order to optimize assay sensitivity and consistency among procedures. These assay conditions could therefore be widely used to elucidate precisely the role of ghrelin isoforms on eating behavior in physiological and pathological situations.

1. Introduction

Preproghrelin is a complex gastrointestinal prohormone which produces two isoforms, acylated (AG) and desacylated (DAG) ghrelin. AG is the endogenous agonist of the Growth Hormone Secretagogue Receptor (GHS-R). It displays pleiotropic effects, being a powerful growth hormone secretagogue and orexigenic peptide with an ultradian pattern of secretion that is increased in anticipation of meals in healthy humans and rodents [1,2]. AG also plays a key role in reinforcing and motivational aspects of food [3]. Conversion of AG to DAG in blood is dependent on esterase activities while the enzyme Ghrelin-O-Acyl-Transferase (GOAT) catalyzes ghrelin acylation [1,4,5]. High fasting plasma AG and DAG concentrations were reported in patients with restrictive-type anorexia nervosa (AN-R) [6], a psychiatric condition characterized by a compulsive self-restriction of food intake. Recent genome wide association studies also demonstrated genetic associations of metabolic traits with AN suggesting an uncovered role of metabolic sensors of undernutrition in the pathophysiology of AN [7]. Although most studies focused on deciphering AG physiological role, both AG and DAG regulate feeding, physical activity and energy metabolism but they have distinct and sometimes opposite actions [8-10]. Higher plasma DAG in AN-R has been recently correlated with reward dysfunctions but in

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sampling conditions that did not allow proper assessment of AG [11].
Therefore, deciphering the specific interaction of ghrelin isoforms is
essential to help elucidating which metabo-psychiatric mechanisms
contribute to AN-R. However, the rapid action of blood esterases impairs
a correct estimation of the AG/DAG ratio [1,12]. The validation of
reproducible methods to collect, process and store human blood samples
to assess AG and DAG plasma variations in clinical studies is still
pending. Furthermore, the limited utilization of sensitive and selective
immunoassays for ghrelin isoforms has been until recently a limitation
to the accurate determination of these ghrelin isoforms in clinical
studies. Finally, most studies assess ghrelin after an overnight fast
without taking into account its physiological pattern of secretion in
the course of a meal. Thus, validated sampling and assay methods to assess
ultradian variations of ghrelin isoforms in relationship with meal and
appetite are critical for a better understanding of their relevance in
pathological eating.

The aim of this study was (1) to compare different methods of
collection, processing and storage of blood samples in healthy women
and (2) apply these conditions to study the ultradian variations of
ghrelin isoforms in relationship with the nutritional state, meal patterns
and meal-associated insulin surge in AN-R patients, in conditions that
are compatible with a clinical practice.

2. Material and methods

2.1. Subjects

Study 1. Evaluation of optimal conditions of sampling, processing
and storage of human blood for validation of ghrelin immunoassays.
Healthy female subjects (HS) were recruited in the Centre de Recherche
Clinique (GHU Paris). Blood samples were withdrawn either after an
overnight fast (9:00 h) or before lunchtime (12:00 h).

Study 2. Impact of nutritional status on longitudinal variations of AG
and DAG in female patients with restrictive-type AN (AN-R). Patients
attended a structured in-patient program in the Eating Disorders Unit of
Clinique des Maladies Mentales et de l’Encéphale (CMME, GHU Paris).
Blood samples were performed in undernourished conditions (acute
phase of the disorder) and after complete weight recovery (100%
reached target BMI, i.e. range between 18.5 and 25), either after an
overnight fast thereby providing baseline morning concentrations (n =
13 patients) or around lunchtime (n = 3 patients). Motivation to eat and
hunger were evaluated with a validated visual analogue scale. The study
protocols were approved by Comité de Protection des Personnes Ile de
France III (EUDRACT N°: 2008-A008 17–48; CPP N° Am5355-2-2592;
CPP 19.07.26.54412) (See Supplementary file for details). Written
informed consent was obtained from all participants before recruitment.

2.2. Conditions of sampling, processing and storage of blood samples

Blood samples were collected in tubes containing 15% EDTA and
supplemented with different protease inhibitors: p-hydroxymercuro-
ibenzoic acid 1 mM (PHMB, a cystine protease inhibitor), Aprotinin 250
KIU (a selective serine protease inhibitor) or 4-(2-aminophenoxy)benzene
sulfonyl fluoride hydrochloride 0.2–2 mg/ml (AEBSF, an irreversible
serine protease inhibitor). Blood samples were placed on ice after
withdrawal then immediately centrifuged at 4 °C (1000 g during 15
min) unless otherwise stated. The impact of delayed centrifugation times
(immmediate, 15, 30, 60, 120 and 180 min after) on acylation preserva-
tion following blood withdrawal was also assessed. In addition, plasma
samples were aliquoted and supplemented or not with HCl 0.1 N or 0.2 N
immediately after collection, then stored at either –20 °C or –80 °C.
Plasma were assayed within 3 months following collection, unless
otherwise stated. Finally, short-term (8 days) versus long-term (447
days) freezing conditions were compared (See Supplementary file for
details).

2.3. Hormone immunoassays

AG and DAG concentrations were assayed with selective two-sites
sandwich enzyme-immunoassays (human AG and DAG Easy Sampling
Elsa kits, Ref A05306 and Ref A05319, respectively, Bertin Bioreagent,
Montigny-le-Bretonneaux, France), either in the condition of one-step
(sample or standard incubated with anti-ghrelin AChE tracer) [9] or
sequential (sample or standard incubated on the plate with a washing
step before adding the anti-ghrelin AChE tracer) protocol. Insulin was
assayed using a competitive enzyme-immunoassay (Ref A05322, Bertin
Bioreagents, Montigny-le-Bretonneaux, France) (See Supplementary file
for details).

2.4. Statistical analysis

Values are given as Mean ± SEM. Statistical analyses were performed
using Student t-test or 1-way or 2-way ANOVAs followed by Tukey’s
post-hoc analysis using GraphPad Prism (GraphPad software). Data
were tested for normality and p values < 0.05 were considered
significant.

3. Results

3.1. Effect of blood treatment and processing, and plasma acidification
and storage conditions on ghrelin stability

3.1.1. Protease inhibitor and HCl supplementation

We first evaluated the stability of ghrelin under various conditions
comparing the concentration of AG, DAG and the AG/DAG ratio (Fig. 1
(See also Supplementary file, section 1.4, for the description of the
protocol used for each experimental data). Treatment of plasma samples
with HCl 0.1 N increased AG over DAG concentrations (ANOVA effect:
AG: p < 0.001; DAG: p = 0.0013) and AG/DAG ratio (p < 0.0001)
regardless of the inhibitors (no HCl x Protease inhibitor interaction)
using either one-step assay (Fig. 1A) or sequential assay (Fig. 1B
and Supplementary Table 1). Under HCl 0.1N-treated conditions, regardless
of the type of assay (one-step vs sequential), results showed no signifi-
cant effect of protease inhibitors on AG concentrations (Fig. 1A, p =
0.46; Fig. 1B, p = 0.12) but increased AG/DAG ratio (p = 0.0002) due to
lower DAG concentrations (p = 0.0013). Post-hoc analysis revealed a
significant increase of AG/DAG ratio in aprotinin conditions compared
to no inhibitor or PHMB (p < 0.05) (Supplementary Table 1).

3.1.2. HCl concentrations

Furthermore, higher HCl concentrations (0.2 N) did not increase AG
or DAG concentrations compared to HCl 0.1 N. Indeed, HCl 0.2 N
significantly reduced AG compared to HCl 0.1 N (p < 0.05) and DAG
concentrations compared to non-HCl treated conditions (p < 0.05), as
well as the AG/DAG ratio (p < 0.05) (Fig. 1C and Supplementary
Table 1).

3.1.3. One-step versus sequential assay

Using aprotinin-treated conditions, AG and DAG were similar
and AG/DAG ratio was slightly higher in the sequential compared to the
one-step assay (p = 0.04) (Fig. 1D and Supplementary Table 1).

3.1.4. Delay of centrifugation

Delayed centrifugation of samples had no significant effect on DAG
concentrations nor AG/DAG ratio over time (Supplementary Table 1).
For AG, although repeated analyses showed an overall significant effect
of time on AG concentrations only (p = 0.028), comparison between
each time point was not significant (Fig. 1E).

3.1.5. Duration of freezing

Samples treated with aprotinin and HCl 0.1 N were assayed imme-
diately, after 8 days at –20 °C or after 18 months at –80 °C. Immediate
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assay did not increase AG, DAG nor AG/DAG levels compared to frozen samples (not shown). Thus, −80 °C seemed to be an appropriate sample conservation procedure. Moreover, long-term conservation at −80 °C did not significantly reduce AG nor DAG level (8 days versus 18 months conservation AG: p = 0.11, DAG: p = 0.14, AG/DAG: p = 0.76) (Fig. 1F). With AEBSF, however, a reduction in AG/DAG ratio was observed over time (not shown).

3.2. Plasma AG and DAG variations with nutritional state in anorexia nervosa and ultradian rhythmic variations around a meal in three patients

We next measured AG and DAG plasma concentrations and AG/DAG ratio in 13 patients with AN-R under different nutritional status (undernourished or refed) and during the course of a meal in 3 different patients (Fig. 2). Blood was collected on EDTA coated tubes supplemented with aprotinin, centrifuged within 15 min, plasma were acidified with HCl 0.1 N and stored at −80 °C prior to performing immunoassays in conditions of one-step. In the undernourished state (IMC = 15.0 ± 0.3), patients showed significantly greater levels of AG (p = 0.0039) and DAG (p = 0.0219) compared to refed state (IMC = 20.1 ± 0.1) (Fig. 2A). Furthermore, decreased AG and DAG concentrations coincided with the occurrence of a post-prandial insulin peak and was associated with a reduction in hunger and motivation to eat in the course of a meal in three patients with AN-R (Fig. 2B–F).

4. Discussion

Ghrelin is a hormone highly sensitive to nutritional status but the exploration of its ultradian variation is limited by non-standardized assay techniques. To our knowledge, only a few studies compared ghrelin assay procedures in humans providing partial results, mainly focusing on AG in non-clinical cohorts. For example, Trivedi et al. [13]
tested various protease inhibitors but not acidification of samples; Blatnik et al. [12] used assay methods insensitive to low DAG levels and Delhanty et al. [14] compared various conservation techniques using only AEBSF as a protease inhibitor. The present study provides a methodology for blood sampling and processing, storage conditions and immunoassay procedures compatible with routine clinical practices combining high feasibility and high relevance to assay both ghrelin isoforms. We advise collecting blood on a tube containing EDTA and keep samples on ice to limit AG degradation [1]. We recommend centrifugation of blood samples as soon as possible but ghrelin is stable up to 180 min when blood is conserved on ice, facilitating routing to a technical platform. Acidification of centrifuged plasma before storage increases AG recovery and lowers ghrelin degradation without affecting total ghrelin concentrations, regardless of the protease inhibitor used. Acidification and protease inhibition (amongst other sample processing) was previously described in the RAPID (“Reduced temperatures, Acidification, Protease inhibition, Isotopic exogenous controls, and Dilution”) method. It increased AG’s recovery over total ghrelin compared to standard procedures in rodents (i.e. EDTA-blood on ice) [15]. In the present study, the AG/DAG ratio was increased by 5-fold when blood was treated with EDTA + aprotinin and by 17-fold when plasma was supplemented with HCl compared to EDTA conditions only.

Treatment with HCl inhibits the activity of butyrylcholinesterase, a major esterase known as a key factor of ghrelin degradation [1]. Adjunction of a protease inhibitor (PI) is also preferable and not all PI are equivalent. AEBSF inhibits a cholinesterase inhibitor and interacts with the tracer AChE, thus requires sequential assay (i.e. incubation with sample or standard alone followed by incubation with anti-ghrelin AChE tracer). This heavier procedure did not show significantly different results between AEBSF and aprotinin neither with a one-step assay using aprotinin. Moreover, aprotinin slightly increased AG/DAG ratio compared to other inhibitors, both after short-term or long-term storage, enabling us to get rather high AG/DAG ratio around 1.5 in the one-step assay. These results are consistent with previous studies, suggesting that aprotinin is the optimal protease inhibitor to our point of view [14]. In such conditions, samples can be kept for various months at ~80 °C with a limited impact, which is convenient in case of longitudinal follow up studies as, for example, explorations in AN-R patients at multiple time-points over the course of renutrition. The sandwich assay used in the present study presents two advantages for clinical studies. Firstly, it has been validated using HPLC and mass spectrometry and demonstrates high specificity for AG and DAG [16] and secondly, its sensitivity allows to use minimal volumes of plasma within serial sampling protocols particularly necessary in undernourished patients. Possible limitations of the current study are that it does not include systematically the same individuals in all different experimental comparisons, likely increasing the variability in AG/DAG ratio. Indeed, the AG/DAG ratio varies considerably (from 0.02 to 0.5 in non HCl-treated conditions and from 0.5 to almost 3 in HCl-treated conditions). Within a set of samples that are collected, processed and assayed exactly in the same conditions (See Supplementary Fig. 1), the most probable explanation is that this reflects biological variability in individual subjects.

An unusual limitation to the study of ghrelin role in pathophysiological conditions is its ultradian mode of secretion [1,17]. We here report for the first time that AG and DAG follow a rhythmic pattern of secretion in anticipation of meals in a case study of three patients with AN-R, similar to previous results on healthy subjects [1]. Furthermore, the variations of AG and DAG can be precisely assessed within a longitudinal perspective and around a meal. Such validated sampling and assay conditions enable the detection of ghrelin variations around mealtime in AN-R patient under undernourished and refed conditions, crucial for clinical research devoted to eating disorders. Although we only provide here a proof of concept that ghrelin rhythmic variations can be detected in 3 different AN patients, using our methodology, evaluation in a sufficient number of patients will be necessary to confirm this pattern and for comparative analysis between undernourished and refed conditions.

To conclude, technical improvements in ghrelin assays may open new windows for routine applications in clinical research. The significance of ghrelin variations in correlation with meal patterns and behavioral responses need to be further assessed to elucidate the complex interaction between metabolic sensing and clinical phenotypes of patients with AN.

Author contributions

Tezenas du Montcel C: Investigation, Visualization, Formal analysis, Writing -original draft & editing. Duriez P: Conceptualization, Investigation. Lebrun N: Data curation, Validation, Formal analysis. Grouselle D: Investigation, Methodology, Data curation. Degrimaudet B: Validation, Formal analysis. Dardenness R: Resources, Investigation. Epelbaum J: Conceptualization, Writing – Review & Editing. Cuenga M: Resources, Data curation. Viltart O: Writing – Review & Editing. Gorwood P: Resources, Funding Acquisition, Supervision. Tolle V: Conceptualization, Project administration, Methodology, Supervision, Writing – Review & Editing.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Benoît de Grimaudet is working at Bertin Biotechnologies.

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