European Review for Medical and Pharmacological Sciences 2020; 24: 1585-1589

Understanding plasma treatment effect on human acyl-ghrelin concentrations

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Abstract. – OBJECTIVE: We evaluated the effect of different concentrations of the esterase inhibitor, AEBSF, and acid treatment on acyl-ghrelin stability in human plasma samples subjected to a freeze/thaw cycle.

MATERIALS AND METHODS: Four plasma samples were collected from each donor and treated with the following concentrations of AEBSF: 2 mg/ml, 1 mg/ml, 0.6 mg/ml, and 0 mg/ml. For each plasma tube collected, half of the aliquots were treated with HCl and stored at -80°C before measuring acyl-ghrelin concentration using enzyme-linked immunosorbent assay (ELISA).

RESULTS: Treatment with 1 mg/ml AEBSF + HCl resulted in significantly higher acyl-ghrelin levels compared to all other treatments except 2 mg/ml AEBSF + HCl or 0.6 mg/ml AEBSF + HCl. While all HCl-treated samples had higher acyl-ghrelin levels than their AEBSF-matched un-acidified samples, only samples treated with 1 mg/ml AEBSF significantly differed in acyl-ghrelin levels as a result of HCl treatment.

CONCLUSIONS: Our results suggest the use of 1 mg/ml AEBSF with HCl for optimal acyl-ghrelin stability in human plasma samples subjected to a freeze/thaw cycle before assay. Given that 2 mg/ml and 0.6 mg/ml AEBSF + HCl did not significantly differ from 1 mg/ml AEBSF + HCl, our data suggest that the use of AEBSF with HCl more potently prevents de-acylation of ghrelin than either treatment alone.

Key Words:
Biomarkers, Clinical, ELISA, Immunoassay, Metabolism.

Introduction

Ghrelin is a 28 amino acid peptide hormone that acts as the endogenous ligand of the growth hormone secretagogue receptor 1a (GHS-R1a, also known as the ghrelin receptor) and aids in the control of energy metabolism through regulation of growth hormone secretion, hunger, and gastric and pancreatic acid secretion[1]. These known functions have made ghrelin a point of interest in the study of diseases involving dysregulation of energy metabolism, such as diabetes mellitus, cachexia, eating disorders, obesity, and alcohol and substance use disorders and have consequently increased interest in measuring ghrelin levels in clinical samples[2]. Ghrelin is primarily secreted from the enteroendocrine X/A-like cells of the stomach[3,4], with secondary sights of expression including the small intestine, colon, lung, heart, kidney, testis, pituitary, and hypothalamus[5-9]. Upon synthesis, ghrelin undergoes acylation of the hydroxyl group at its serine-3 residue through the enzyme, ghrelin O-acyltransferase. Ghrelin O-acyltransferase co-localizes with ghrelin in gastric X/A cells, where it uses acyl groups derived from fatty acids in the stomach to acylate ghrelin before it is released into circulation. Upon acylation, ghrelin is referred to as acyl-ghrelin (other terms: active ghrelin, ghrelin), and acts as the endogenous agonist to GHS-R. However, circulating acyl-ghrelin can become quickly de-acylated by plasma esterases, such as butyrylcholinesterase (BuChE), creating a form of ghrelin referred to as des-acyl ghrelin (other terms: inactive ghrelin)[10]. The physiological functions of des-acyl-ghrelin, if any, remain yet unclear but appear to be antagonistic to acyl-ghrelin[11]. However, unlike the recently discovered endogenous GHS-R antagonist, liver-expressed antimicrobial peptide (LEAP-2), des-acyl ghrelin does not bind to GHS-R[12]. The rapid metabolism of acyl-ghrelin makes its study difficult. Due to the sensitivity of the O-octanoyl (or rarer decanoyl) moiety to enzymatic degradation, improper blood sample collection and processing can confound subsequent acyl-ghrelin measure-

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ments. Acidification, low temperature, and different enzymatic inhibitors can be used to prevent de-acylation of ghrelin as a result of sample handling, yet the optimal combination of these factors is not clear. Several studies have been conducted to address a lack of knowledge regarding best practices for the collection and preservation of plasma samples for acyl-ghrelin measurement. Blatnik et al[13,14] have demonstrated that the use of the enzymatic inhibitor 4-benzenesulfonyl fluoride hydrochloride (AEBSF) to increase acyl-ghrelin stability in plasma has greater efficacy in reducing acyl-ghrelin metabolism than inhibitors used in other studies, such as heparin and aprotinin[15]. However, the results from Blatnik et al[13,14] also suggest a departure from current commercial human acyl-ghrelin ELISA kit recommendations, which promote the use of 50 mM HCl and 1 mg/ml AEBSF plasma sample treatment[16]. Instead, Blatnik et al[13,14] suggests that a saturating concentration of AEBSF (2 mg/ml) without HCl treatment produces optimal acyl-ghrelin stability in plasma. We therefore decided to conduct an experiment to determine whether the recommendations suggested by Blatnik et al[13,14] should be followed, or adherence to commercial ELISA kit recommendations should be followed in future studies.

Materials and Methods

Materials

Commercial human acyl-ghrelin ELISA assays (EZGRA-88K) and AEBSF hydrochloride (Pefabloc® SC) were both purchased from MilliporeSigma (Burlington, MA, USA).

Participants

Blood was collected from healthy volunteers who remained anonymous to laboratory researchers through the National Institutes of Health (NIH) Department of Transfusion Medicine Research Support Program. This program provides clinical sample donations in support of laboratory research to interested scientists via the NIH Clinical Center protocol 99-CC-0168 ("*Collection and distribution of blood components from healthy donors for in vitro research use*"). In order to enroll in this protocol, healthy volunteer donors must meet the eligibility criteria for volunteer whole blood donation (with the exception of foreign travel history and other requirements). Furthermore, they need to be at least 18-years-old, weigh more than 110 pounds, and have no history of known heart, lung, kidney disease, bleeding disorders, or sickle cell disease. Pregnancy is also an exclusion criterion. For a full list of the inclusion and exclusion criteria and for additional details, please see: https://clinicaltrials.gov/ct2/show/NCT00001846. All participants were provided with a written consent form and informed consent was given before participation in the study. The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the appropriate NIH Institutional Review Board. This specific project on acyl-ghrelin was reviewed and approved by the NIH Clinical Center Department of Transfusion Medicine (DTM) via the New Investigator Registration Approval Renewal System (NARS).

Blood Collection and Sample Processing

For each participant, blood was collected from the forearm vein into 4 mL K_2 EDTA plasma tubes containing 2 mg/ml AEBSF, 1 mg/ml AEBSF, 0.6 mg/ml AEBSF, and 0 mg/ml AEBSF respectively. Samples were placed immediately on ice for transport, and centrifuged for 15 min at 2,000 x g at 4°C. Plasma was then extracted from each tube and separated into aliquots. Half of the aliquots from each tube were treated with 5% HCl (refers to 5% 1 M HCl for final concentration of 0.05 M throughout manuscript) with the remaining tubes left un-acidified. Samples were stored at -80°C until assayed.

Enzyme-Linked Immunoassay (ELISA)

Before assay, plasma samples were thawed on ice water, vortexed, and centrifuged at 2000 x g for 10 min at 4°C to separate lipid content. Acyl-ghrelin levels were then measured via sandwich ELISA according to manufacture instructions. Briefly, assay buffer was added to each well, followed by 20 µL serum matrix to blank, standard, and quality controls. 20 µL of standards and quality controls were added to plate in duplicate and samples were added in triplicate. 50 µL of a 1:1 mixture of capture and detection antibody was added to the wells and the plate was allowed to incubate with shaking (450 rpm) at room temperature for 2 h. Plates were washed with wash buffer 3X using an automated plate washer (TECAN, Männedorf, Switzerland). 100 µL of strepdtavidin-horseradish peroxidase (HRP) conjugate solution was added to the plate and allowed to incubate with shaking at room temperature for 1 h. Plates were washed 6X with buffer before adding 100 µL TMB solution to each well and incubated with shaking for 30 min. 100 µL 0.3 M HCl was added to each well, and absorbance was read at 450 nm using a Promega GloMax microplate

reader (Madison, WI USA). All wells were corrected for non-specific binding by subtracting the absorbance reading from "blank" wells containing only serum matrix. Standard curves were fit using a 4 or 5 parameter logistic equation in Graph Pad Prism version 8 for Windows (Microsoft, Albuquerque, NM, USA). Samples containing a % CV greater than 15% were not included in the analysis. Intra- and inter-assay % CV were 8.13% and 7.9%, respectively.

Statistical Analysis

Data were normalized to the largest average acyl-ghrelin level for each subject to control for between-subject differences in baseline acyl ghrelin levels. Normality was assessed using the Shapiro-Wilk test, and data were evaluated for effective pairing using Pearson's correlations with one-tailed Student *t*-tests. For significantly effective pairs, data were compared using two-tailed paired *t*-tests or Wilcoxon match rank paired sign tests for normally and non-normally distributed data, respectively. For non-significantly effective pairs, normally and non-normally distributed data were compared using two-tailed, unpaired *t*-tests or Mann-Whitney U tests, respectively. Data are expressed as Means ± SE and significance was set at $p < 0.05$. All statistics were applied using GraphPad Prism version 8 software (Microsoft, Albuquerque, NM, USA).

Results

Samples were collected from 5 participants in total. All acidified plasma samples had a higher average acyl-ghrelin concentration in comparison to un-acidified plasma samples treated with the same concentration of inhibitor. However, there was only a statistically significant difference between samples treated with 1 mg/ml AEBSF *vs.* 1 mg/ml AEBSF + 5% HCl (19% reduction in acyl-ghrelin levels; $p = 0.03$) (Figure 1). Moreover, acyl-ghrelin concentration was significantly lower in comparison to 1 mg/ml AEBSF + HCl treatment for all treatments except 2 mg/ml AEBSF + 5% HCl and 0.6 mg/ml AEBSF + 5% HCl, the latter of which only reached trend-level significance ($p = 0.06$) (Figure 1). Specifically, there was a significant difference between 1 mg/ml AEBSF + 5% HCl and the following treatment groups: 2 mg/ml AEBSF (22% reduction in acyl-ghrelin levels; $p = 0.03$), 0.6 mg/ml AEBSF (60% reduction in acyl-ghrelin levels; $p = 0.02$), 0 mg/ml AEBSF (62% reduction in acyl-ghrelin levels; $p = 0.02$), and 0 mg/ml AEBSF + 5% HCl (53% reduction in acyl-ghrelin levels; $p = 0.02$).

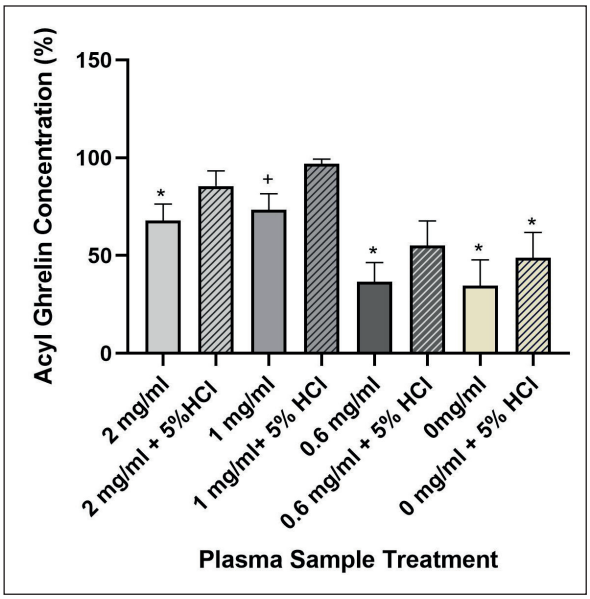

Figure 1. Plasma acyl ghrelin concentrations. Data are expressed as Mean ± SE. Acyl-ghrelin levels are normalized to largest acyl-ghrelin value per subject, therefore, values represent mean percentage of largest acyl-ghrelin concentration per subject across each treatment condition. *$p<0.05$ (*vs.* 1 mg/ml AEBSF + 5% HCl), + $p<0.05$ (*vs.* AEBSF-matched control).

Discussion

Our results support the treatment of plasma samples with 1 mg/ml AEBSF + 5% HCl before storage and assay for acyl-ghrelin concentrations, as currently suggested by the commercial manufacturer of the assay used by our laboratory and Blatnik et al[13,14,16]. We found a significant loss of average acyl-ghrelin values for all un-acidified samples and controls in comparison to 1 mg/ml + 5% HCl treated samples. While samples treated with 2 mg/ml AEBSF + 5% HCl and 0.6 mg/ml AEBSF + 5% HCl were not significantly different from 1 mg/ml AEBSF + 5% HCl, they had an approximate loss of 22% and 35% acyl-ghrelin in comparison, respectively. For comparisons between HCl-treated samples and their AEBSF concentration-controlled counterparts, all acidified samples had a higher mean average of acyl-ghrelin levels, but there was only a significant difference between 1 mg/ml AEBSF treated groups.

Our data appear to differ from that of Blatnik et al[13,14] by demonstrating a significant difference

in acyl-ghrelin stability among samples treated with 1 mg/ml AEBSF + 5% HCl in comparison to 2 mg/ml AEBSF without HCl. While our data find that there isn't any significant difference between 1 mg/ml AEBSF and 2 mg/ml AEBSF when they undergo the same acid treatment (both 5% HCl or 0% HCl), we did observe that there was a slight reduction in acyl-ghrelin levels resulting from the use of 2 mg/ml AEBSF in comparison to 1 mg/ml AEBSF. The 2 mg/ml AEBSF dose has been used with the estimate that AEBSF would be present in approximately 100,000 fold molar excess to BuChE in blood and plasma, assuming 4 μg/ml BuChE in circulation[13,14,17]. AEBSF is a sulfonyl fluoride that irreversibly binds to serine proteases, yet it is also reactive to threonine, lysine, tyrosine, histidine, and cysteine residues (for brief review, see[18]). Therefore, it has been suggested that the use of high concentrations of AEBSF can result in the formation of protein adducts. This raises the possibility that the formation of protein adducts can disrupt sandwich assays of target proteins containing these residues, and may explain the slight reduction of acyl-ghrelin stability observed in comparison to a lower (1 mg/ml) concentration of AEBSF. We saw a decrease in acyl-ghrelin stability resulting from yet lower (0.6 mg/ml, 0 mg/ml) concentrations of AEBSF, suggesting that 1 mg/ml AEBSF produces optimal acyl-ghrelin stability. However, we would like to note that these observed differences were not statistically significant. Significant differences in acyl-ghrelin levels were observed between 1 mg/ml AEBSF + 5% 1M HCl *vs.* 1 mg/ml AEBSF treated samples. The use of acidification for sample preservation is a common technique adapted to reduce the charge environment in plasma, thereby reducing enzymatic activity. Indeed, 10% HCl (100 mM) has been suggested based on results of the study of synthetic acyl-ghrelin using RIA, and the study of human acyl-ghrelin using internally developed sandwich ELISAs[15,19,20]. In contrast, Blatnik et al[13,14] have suggested that 50 mM or 100 mM HCl does not appreciably alter acyl-ghrelin stability. In this study, we observed a significant increase in acyl-ghrelin stability as a result of 50 mM HCl treatment in 1 mg/ml AEBSF samples, and a non-significant increase in mean acyl-ghrelin concentration as a result of HCl treatment among all other samples, including controls. One possible reason for this difference may be due to the fact that our samples were stored in a -80°C freezer prior to assay, whereas samples used for HCl-treatment comparisons in Blatnik et al[13,14] were assayed immediately. Therefore, the protective effects of HCl on acyl-ghrelin stability may become more apparent when samples are subjected to freeze/thaw cycles, where lower pH can prevent hydrolysis of AEBSF and rapid de-acylation of ghrelin upon thawing. Indeed, increased stability of the O-octanoyl moiety of acyl-ghrelin as a result of HCl has been demonstrated in plasma samples spiked with synthetic ghrelin and subjected to multiple freeze thaw cycles[15]. Therefore, acidification may be a particularly important step in clinical studies, where freeze/thaw cycles are often necessary due to the need for freezer storage during participant accrual. In summary, our results provide support for the use of 1 mg/ml AEBSF + 5% HCl human sample treatment in the measurement of plasma concentrations of acyl-ghrelin.

Conclusions

The work presented here provides further insight into optimal measurement of acyl ghrelin from plasma samples. While previous reports suggest a departure from commercial recommendations regarding concentration of AEBSF and acidification, our results preliminarily support commercial guidelines by showing increased acyl ghrelin stability using 1 mg/ml AEBSF and 50 mM HCl. Moreover, our study suggests that the effects of HCl on acyl-ghrelin stability may become more apparent when samples undergo freeze/thaw cycles before assay.

Acknowledgments

The authors would like to thank Kamille West, M.D. and Tom Lewis (NIH Clinical Center Department of Transfusion Medicine) and Erick Singley (Clinical Core Laboratory, Office of Clinical Director, NIAAA) for administrative and technical support. The authors would also like to thank Diane Cooper (NIH Library) for bibliographic assistance.

Funding

This work was supported by: 1) National Institutes of Health (NIH) intramural funding ZIA-AA000218 (Section on Clinical Psychoneuroendocrinology and Neuropsychopharmacology; PI: Dr. Lorenzo Leggio), jointly supported by the NIAAA Division of Intramural Clinical and Biological Research and the NIDA Intramural Research Program: 2) the NIH Department of Transfusion Medicine (DTM) Research Blood Program.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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