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PII: S0024-3205(22)01005-0

DOI: https://doi.org/10.1016/j.lfs.2022.121305

Reference: LFS 121305

To appear in: *Life Sciences*

Received date: 19 October 2022

Revised date: 6 December 2022

Accepted date: 14 December 2022

Please cite this article as: A.S. Fittipaldi, D. Castrogiovanni, D. Lufrano, et al., Ghrelin proteolysis increases in plasma of men, but not women, with obesity, *Life Sciences* (2022), https://doi.org/10.1016/j.lfs.2022.121305

Ghrelin Proteolysis Increases in Plasma of Men, but not Women, with Obesity

Running title: ghrelin proteolysis in human obesity

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Running title: ghrelin proteolysis in human obesity

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Abstract

Aims: Since plasma ghrelin can undergo des-acylation and proteolysis, the aim of this study was to investigate the extent to which an enhancement of these reactions is associated to the decrease of ghrelin in plasma after food intake or in individuals with obesity.

Main methods: we performed an intervention cross-sectional study, in which levels of ghrelin, desacyl-ghrelin (DAG), glucose, insulin, ghrelin des-acylation and ghrelin proteolysis were assessed in plasma before and after a test meal in 40 people (n=21 males) with normal weight (NW, n=20) or overweight/obesity (OW/OB, n=20).

Key findings: Preprandial ghrelin and DAG levels were lower, whereas preprandial ghrelin proteolysis was ~4.6-fold higher in plasma of males with OW/OB. In males, ghrelin proteolysis positively correlated with glycemia. Ghrelin and DAG levels were also lower in females with OW/OB, but preprandial ghrelin proteolysis was not different between females with NW or OW/OB. Ghrelin and DAG levels decreased postprandially in males and females, independently of BMI, and ghrelin proteolysis increased postprandially ~2 folds only in individuals with NW. Ghrelin des-acylation remained unaffected by BMI or feeding status in both sexes.

Significance: Current study shows that ghrelin proteolysis increases in males with obesity as well as after meal in lean individuals. Therefore, ghrelin proteolysis may be an important checkpoint and, consequently, a putative pharmacological target to control circulating ghrelin levels in humans.

Key words: ghrelin proteolysis, ghrelin des-acylation, obesity.

Introduction

Ghrelin is a 28-residue peptide hormone predominantly secreted by cells of the stomach that displays several unique biochemical and biological features [1]. Chemically, ghrelin is the only peptide known to be acylated with an octanoic moiety, a posttranslational modification that takes place at Ser3 of ghrelin and is catalysed by the enzyme ghrelin-O-acyltransferase (GOAT) early in the biosynthetic processing within the ghrelin-producing cells [2]. The biological actions of ghrelin, which are mediated by the growth hormone secretagogue receptor (GHSR), entirely depend on the octanoylation of ghrelin and include the most potent orexigenic effect described to date [3,4]. Ghrelin also induces growth hormone and glucocorticoid secretion, recruits several mechanisms that increase glycemia, increases gastrointestinal motility and affects non-homeostatic aspects of food intake, among other effects [1]. Thus, ghrelin is a pleiotropic hormone that contains an exceptional posttranslational modification that is essential for its bioactivity.

Plasma ghrelin level is tightly regulated by the daily feeding status and by the long-term energy balance. In the short term, ghrelin level increases before meals and decreases to baseline levels within the first hour after meals [5]. Ghrelin level increases in prolonged energy deficit conditions, such as fasting, calorie restriction or anorexia nervosa [6,7], when a rise in plasma ghrelin contributes to seek for food and maintain glycemia. In contrast, ghrelin level decreases under energy surplus conditions, such as obesity [8,9], and such decrease presumably results as a compensatory adaptation aiming to reduce feeding and glycemia. As other hormones, plasma ghrelin level depends on the equilibrium among its secretion, degradation and clearance rates. A large body of studies characterized the mechanisms controlling the biosynthesis and release of ghrelin from ghrelin cells mainly using animal models or *in vitro* systems [10]. Conversely, the molecular mechanisms controlling the disappearance of ghrelin from the circulation have been less investigated. Ghrelin rapidly disappears from plasma, having a half-life of 9-13 min after a single bolus injection of the hormone [11-13], and the key chemical reactions affecting plasma ghrelin involve not only des-acylation but also proteolysis. Ghrelin des-acylation in plasma results in the generation of desacyl-ghrelin (DAG), which is also secreted from ghrelin cells. DAG does not bind to GHSR at physiological levels, but it has been suggested to display some GHSR-independent effects on insulin secretion, osteoblast growth and lipid metabolism [1]. Ghrelin des-acylation in plasma is presumably mediated by different esterases including platelet activating factor acetylhydrolase, paraoxonase, α2-macroglobulin,

butyrylcholinesterase and acyl protein thioesterase 1; although it is likely that other esterases can also hydrolyze the lipid moiety from ghrelin [14-19]. Ghrelin also undergoes proteolysis in plasma giving rise to shorter peptides [15,16]. Ghrelin proteolysis mainly occurs at the middle segment of the peptide (i.e. between Arg15 and Lys16) and generates peptides of different length, including peptides containing the N-terminal end of ghrelin that may be able to evoke some activation of GHSR although less potently than full-length ghrelin [16,20,21]. Ghrelin proteolysis was suggested to involve endopeptidase activated protein C in bovine plasma [22], and insulin-degrading enzyme in an *in vitro* system [23]. Still, the molecular identity of the enzymatic system mediating ghrelin inactivation in plasma remains to be fully clarified.

Since its discovery, manipulating ghrelin system emerged as a putative strategy in the treatment of obesity and related diseases. In order to further reduce ghrelin actions in obesity and achieve additional beneficial effects, GHSR antagonists, ghrelin vaccines and GOAT inhibitors have been developed; however, none of these strategies reached the desired effects in human trials [24]. Notably, the inhibition of the extracellular inactivation of incretins has proved to be a critical strategy to improve glycaemic control in patients suffering type 2 diabetes [25] . In this context, understanding the molecular mechanisms controlling the level of ghrelin in plasma emerges as a fundamental step forward to not only better understand the biology of ghrelin but also find new pharmacological strategies to manipulate such levels and achieve beneficial effects. To the best of our knowledge, the extent to which des-acylation or proteolysis of ghrelin in plasma are affected by the daily feeding status or obesity has not been previously investigated. Here, we hypothesized that decrease of plasma ghrelin in individuals with obesity and/or in the postprandial state could be due to an increase in its extracellular processing. In order to test our hypothesis, we performed a single test meal study in individuals with either normal weight (NW) or with overweight or obesity (OW/OB) and assessed not only levels of ghrelin and DAG in plasma but also the specific levels of ghrelin des-acylation and ghrelin proteolysis in plasma.

Materials and Methods

2.1 Study design. The study was approved by the Institutional Ethical Committee of San Martin Hospital, La Plata (Protocol ID HSMLP2020/0044) and conducted according to the Declaration of Helsinki guidelines and Argentinian legal provisions governing clinical research in humans. Written informed consents were obtained from all participants. Participants were recruited via advertising in social media from August to October 2021.

2.2 Study participants. This intervention cross-sectional study included 20–45 years-old adults, without chronic medical illness, personal history of diabetes, history of medical condition, or medication related to obesity or diabetes risk status. The study included 21 males and 19 females. Pregnant women were excluded from the study. Sample size was calculated to unmask differences in plasma ghrelin levels, assuming a standard deviation of 3.26 fmol/ml [26], 80% statistical power and 5% significance level in preprandial ghrelin level, leading to a minimal sample size of 40 participants (20 with NW and 20 with OW/OB).

2.3 *Study protocol*. All participants arrived at the facilities of the Institute in the morning after a 12-h overnight fast and underwent an initial evaluation, including a medical and family history. Weight and body composition were measured with the participants barefoot and wearing minimal clothing, on a bioimpedance scale (Omron HBF514, Argentina) with a resolution of 10 g. Height was measured with a Harpenden-type wall-mounted stadiometer (Holtain Ltd., United Kingdom) with a resolution of 1 mm. BMI was calculated by dividing weight by height squared (kg/m^2). As described by the World Health Organization, participants were classified as NW when BMI was between 18.5 and 24.99 Kg/m^2 or as OW/OB when BMI≥25 Kg/m^2 [27]. Next, a preprandial blood sample was obtained. Participants were given a test meal (breakfast) that provided 20% of the individual's daily energy requirement as estimated using the "Body Weight Planner" digital application of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, USA) [28]. The breakfast composition was 56 kcal% carbohydrates, 12 kcal% protein and 34 kcal% lipids and comprised yogurt, cereals and almonds. Participants were asked to consume the entire breakfast in less than 15 min. Blood sampling was repeated at 60 min after starting breakfast (postprandial), when plasma ghrelin level is significantly reduced, according to a meta-analysis study [5]. Blood samples were collected in tubes containing either heparin, to assess glucose and insulin, ghrelin proteolysis and ghrelin

des-acylation, or EDTA (1 mg/mL final) and p-hydroxy-mercuribenzoic acid (0.4 mM final), to assess ghrelin and DAG. For ghrelin and DAG assays, plasma was immediately acidified with HCl (0.1 N final) [29]. All samples were stored frozen at -80°C until analysis.

2.4 Biochemical analyses. Glycemia was measured using an enzymatic procedure in a CM 250 (Wiener Lab, Argentina). Insulin level was assessed using chemiluminescence (Advia Centaur XP, Siemens, Germany). Insulin resistance was estimated by the homeostasis model assessment for insulin resistance (HOMA index) [30]. Ghrelin and DAG were assessed using specific enzyme immunoassays from Bertin Bioreagent (A05306 and DAG, respectively), and following manufacturer's instructions. Ghrelin/DAG ratio was computed on a molar basis as ghrelin concentration divided by DAG concentration. The food intake-induced change of plasma ghrelin was calculated as preprandial minus postprandial ghrelin concentration in molar basis.

2.5 Assessment of ghrelin des-acylation in plasma. Here, we used a biotinylated analogue of ghrelin consisting in the first 11 residues of ghrelin with the octanoyl moiety linked to Ser3 and a biotin attached to the ε-amino group of Arg (biotin-ghrelin or GSS(Oct)FLSPEHQR-biotin). To assess ghrelin des-acylation in plasma (Fig. 1A), biotin-ghrelin (0.01 µg/µL) was incubated with each plasma sample (20 % v/v) in phosphate buffer saline at 37 ºC. After 60 min, trifluoroacetic acid (20 % final) was added to the tube to stop the reaction. We next used a reverse-phase chromatographic procedure to separate biotin-ghrelin from biotin-DAG, based on a previously described protocol [2]. Briefly, reaction mixtures for each plasma sample were applied to a ZipTip with C18 resin, according to manufacturer's instructions, and eluted with a 20% and 100%-CH_3CN solutions in 0.1% TFA in order to elute biotin-DAG and biotin-ghrelin, respectively. After CH_3CN evaporation, eluted samples were subjected to dot blot using PVDF membranes. Next, blot membranes were incubated with Vectastain Elite ABC kit (Vector Laboratories, cat # PK6200) for 2 h, according to manufacturer's protocols, and the presence of biotin-ghrelin and biotin-DAG was visualized using a 3-3'-diaminobenzidine (DAB)/Nickel solution, which gives a black/purple precipitate. DAB signal per spot was registered using a Gel Doc XR (Bio-Rad) and quantified using the software Fiji. Ghrelin des-acylation was calculated as the ratio between the intensity of biotin-DAG and biotin-ghrelin and expressed as relative units (r.u.). The accuracy of the separation and quantification system was checked using solutions with different concentrations of either intact biotin-ghrelin and biotin-DAG.

2.6 Assessment of ghrelin proteolysis in plasma. Here, we used a fluorescent analogue of human ghrelin (F-ghrelin) consisting in the first 19 residues of ghrelin with the octanoyl moiety linked to a diaminopropanoic acid (Dpr) at position 3 and a fluorescein isothiocyanate attached to the ε-amino group of Lys19 (GSDpr(Oct)FLSPEHQRVQQRKESK(FITC)-NH_2), which was synthesized as previously described [31]. To assess ghrelin proteolysis in plasma (Fig. 1B), F-ghrelin (0.025 µg/µL) was incubated with each plasma sample (12.5 % v/v) in phosphate buffer saline at 37 ºC. After 180 min, loading buffer was added to the tubes and reaction mixtures for each sample were loaded onto 20% polyacrylamide gels prepared according to the Ornstein-Davis discontinuous system. Fluorescence was visualized by trans-UV exposure, registered using a 520DF30 emission filter (Gel Doc XR, Bio-Rad) and quantified using the software Fiji. Ghrelin proteolysis was calculated as the ratio between the fluorescence of the shorter peptide products of cleavage and the fluorescence of intact F-ghrelin, and expressed as relative units (r.u.).

2.7 Statistics and data analyses. Statistical analyses were performed using GraphPad Prism 9.0.0 Normal distribution of data was tested using the Shapiro-Wilk test. Preprandial characteristics in males were compared with Student's unpaired t test or Mann Whitney test. Positively skewed variables (ghrelin, DAG, ghrelin/DAG, and ghrelin proteolysis) were log-transformed for all subsequent analyses. Changes in time between groups were evaluated with two-way repeated measures analysis of variance (ANOVA), with group (NW vs. OW/OB) as between factor and feeding condition (preprandial vs. postprandial) as within factor. Linear correlations between log-transformed ghrelin proteolysis and other variables were assessed using Pearson's (ghrelin, BMI and glycemia) or Spearman's (DAG) correlation coefficients. Tests used for each comparison were indicated in each figure legends. Variables with normal distribution were expressed as the mean ± standard deviation (SD), and variables with non-normal distribution were reported as median (interquartile range [IQR]). Differences were considered statistically significant when $p<0.05$.

Results

3.1 Ghrelin level decreases whereas ghrelin proteolysis increases in preprandial plasma of males with OW/OB. Initially, male participants were grouped as NW (n=9) or OW/OB (n=12). As shown in Table 1, age did not differ between groups nor correlate with any assessed parameter, whereas BMI, preprandial glycemia, preprandial insulin levels and HOMA index were significantly higher in OW/OB group, as compared to NW group. Conversely, preprandial ghrelin (Fig. 2A) and DAG (Fig. 2B) levels were ~2.2- and ~6.0-fold lower, respectively, in OW/OB group, as compared to NW group. Ghrelin/DAG ratio (Fig. 2C) was ~8.5-fold higher in OW/OB group, as compared to NW group, suggesting that the mechanisms controlling plasma ghrelin level are differentially affected in males with OW/OB. Thus, we assessed the capacity of plasma samples to des-acylate a biotinylated ghrelin analogue. We found that ghrelin des-acylation was not different between plasma samples of NW and OW/OB groups (Fig. 2D). Next, we assessed the capacity of plasma samples to proteolyze a fluorescent ghrelin analogue. Here, ghrelin proteolysis was ~4.6-fold higher in OW/OB group, as compared to NW group (Fig. 2E). Notably, ghrelin proteolysis negatively correlated with plasma levels of ghrelin and DAG (Fig. 3A-B), it did not correlate with age (r=0.223, p=0.3317, not shown), plasma insulin levels (r=0.269, p=0.239, not shown), HOMA index (r=0.303, p=0.182, not shown) nor BMI (Fig. 3C) and it positively correlated with glycemia (Fig. 3D).

3.2 Postprandial ghrelin proteolysis in plasma increases in males with NW, but not with OW/OB, whereas postprandial ghrelin des-acylation remains unaffected. After the single test meal intervention, glycemia was not affected by feeding condition (p condition= 0.820), and remained higher in OW/OB group, as compared to NW group (p group=0.012). Plasma levels of ghrelin and DAG changed in a group- and feeding condition-dependent manner, being decreased in OW/OB group and postprandially (Fig. 4A-B). Here, the food intake-induced change of plasma ghrelin level tended to be lower in OW/OB group, as compared to NW group (6.5±5.7 vs. 10.9±7.8 fmol/mL, respectively, P=0.0765). Notably, ghrelin des-acylation remained unaffected by group or feeding condition (Fig. 4C). Ghrelin proteolysis also showed a significant interaction between the effects of group and feeding condition, being ~1.6-fold increased after food intake only in NW group and increased in OW/OB group, independently of the feeding condition (Fig. 4D).

3.3 Postprandial ghrelin proteolysis increases in NW females but remains unaffected in plasma of females with OW/OB, independently of the feeding condition. Since some evidence indicate that ghrelin level fluctuates in a sex-dependent manner in humans [32,33], we also studied females under the same study protocol and grouped them as NW (N=11) or OW/OB (n=8). As shown in Table 2, age and preprandial glycemia did not differ between groups whereas BMI, preprandial insulin levels and HOMA index were significantly higher in OW/OB group, as compared to NW group. In preprandial condition, ghrelin and DAG levels were ~2.0- and ~2.6-fold lower, respectively, in OW/OB group, but ghrelin/DAG ratio was not different between both groups, suggesting that the mechanisms controlling plasma ghrelin level in females with OW/OB were less affected than in males with OW/OB. Here, ghrelin des-acylation and ghrelin proteolysis in plasma were not different between NW and OW/OB groups (Table 2). In contrast to males, ghrelin proteolytic activity in plasma did not correlate with glycemia in females (r=0.154, p=0.530).

After the test meal intervention, plasma levels of ghrelin and DAG changed in a group- and feeding condition-dependent manner, being decreased in OW/OB group and postprandially (Fig. 5A-B). Here, the food intake-induced change of plasma ghrelin level was lower in the OW/OB group, as compared to NW group (10.2±8.3 vs. 26.4±16.75 fmol/mL, respectively, P=0.0230). As found in males, ghrelin des-acylation was unaffected by the feeding condition in NW and OW/OB groups (not shown) whereas ghrelin proteolysis was postprandially increased ~2.2 folds only in NW group (Fig. 5C).

Discussion

Here, we performed a single test meal study in individuals with NW or OW/OB and investigated levels of ghrelin, ghrelin des-acylation and ghrelin proteolysis in plasma. We confirmed that preprandial ghrelin level is lower in individuals with OW/OB, independently of sex, as previously shown [5,8,9,32,34-38]. After food intake, ghrelin level decreased in males and females of NW and OW/OB groups. Interestingly, ghrelin proteolysis was higher in plasma of males, but not females, with OW/OB and correlated with glycemia. Ghrelin proteolysis increased after food intake in individuals with NW only. In contrast, ghrelin des-acylation was similar in plasma of individuals with NW or OW/OB and remained unchanged after food intake.

The current study was carefully designed to assess the chemical reactions affecting plasma ghrelin at two key time points: in the morning after overnight fasting, when plasma ghrelin is around the highest diurnal level [8], and 60 min after meal, when plasma ghrelin decreases [5]. Indeed, we detected the expected postprandial decline in ghrelin level in most lean individuals (19 out of 20), as shown in several previous reports [5,32,35-38], confirming that plasma ghrelin level was studied when it undergoes a significant change. It is important to highlight that it is now well established that the inaccurate manipulation of plasma samples results in the conversion of most ghrelin into DAG and a consequent low ghrelin/DAG ratio [39]. Here, plasma ghrelin/DAG ratio in NW individuals was ~0.6, which is similar to the highest reported ghrelin/DAG ratios in similar studies (see for instance [40]), indicating that the conditions of sampling and storage were accurate to preserve ghrelin in plasma despite the complexity involved in clinical studies. Finally, current study was performed in relatively young individuals in order to minimize the putative impact of the age-dependent decline in plasma ghrelin that has been observed older individuals [41].

In order to specifically assess ghrelin des-acylation or proteolysis in plasma, we set up two reproducible assays whose characteristics should be taken in consideration when interpreting current findings. Importantly, we used two different labelled variants of ghrelin that rely on modifications at its C-terminal end, which is not essential for bioactivity [20,42]. To assess ghrelin des-acylation, we used biotin-ghrelin, a 11-residue analogue of ghrelin biotinylated at the Arg11 C-terminus. Biotin-ghrelin is susceptible to hydrolysis by esterases, since the octanoyl moiety is coupled at Ser3 through an ester bond, but

presumably resistant to plasma proteases, since ghrelin1-11 is the shorter derivative of ghrelin found after proteolysis of full-length ghrelin [22]. To assess ghrelin proteolysis, we used F-ghrelin, an 18-residue analog of ghrelin conjugated to fluorescein isothiocyanate through a Lys added at its C terminus. F-ghrelin contains the entire peptide segment susceptible to proteolysis, according to previous descriptions [22], but is resistant to esterases because Ser3 was replaced with Dpr, which is coupled with the octanoyl moiety through an amide bond [31]. Of note, F-ghrelin and the fluorescent products of proteolysis were directly visualized and quantified after separation by native gel electrophoresis. In contrast, visualization and quantification of biotin-ghrelin and biotin-DAG required several steps including reverse-phase chromatographic procedure for separation, dot-blot and chromogenic staining. Thus, it is possible that the method used to assess ghrelin des-acylation lacked enough sensitivity to unmask small differences in enzymatic activity. Also, reaction times to estimate each enzymatic activity were set up in order to have detectable amounts of substrate and products of each assay. Thus, ghrelin des-acylation and ghrelin proteolysis assays required 60 and 180 min of reaction time, respectively. The observation that the hydrolysis of peptide bonds of ghrelin in plasma is a relatively slow process would suggest that changes in ghrelin proteolysis have stronger implications in long-term conditions, as we observed here in males with OW/OB. In contrast, the physiological implications of the detected postprandial increase of ghrelin proteolysis in plasma of lean individuals is uncertain given the difference between the timing for the rapid postprandial decrease of plasma ghrelin and the reaction time required to assess ghrelin proteolysis in our experimental conditions.

To the best of our knowledge, the current study provides the first indication that proteolysis of ghrelin in plasma increases in males with OW/OB. The observation that ghrelin proteolysis negatively correlates with plasma levels of ghrelin and DAG in the preprandial state suggests that increased ghrelin proteolysis in males with OW/OB may contribute to reduce plasma levels of these peptides. Also, increased ghrelin proteolysis in obesity may contribute to impair the effects of ghrelin treatment (the so-called ghrelin resistance), reported in obese mice [43]. Strikingly, we found here that ghrelin proteolysis positively correlated with glycemia, but did not correlate with insulinaemia or HOMA index. Conversely, preprandial ghrelin proteolysis and glycemia remained unaffected in females with OW/OB, despite they showed reduced preprandial plasma ghrelin level and insulin resistance, as compared to females with NW. Thus, preprandial ghrelin proteolysis is somehow associated to glucose level itself, via mechanisms that do not seem to involve

insulin. The reasons why ghrelin proteolytic activity increases only in males with OW/OB are uncertain, but it is in line with the observation that ghrelin level in obesity tends to be lower in males than females, as found here and reported in previous studies [32,33]. Interestingly, insulin-degrading enzyme level in plasma was found higher in individuals (of undisclosed sex) with metabolic syndrome and strongly correlated with the severity of the symptoms [44]. Also, plasma activated protein C level was higher in individuals (male and female combined) with obesity and reduced after bariatric surgery [45,46]. Thus, some proteases able to cleave ghrelin are strong candidates to mediate increased ghrelin proteolysis in plasma of male individuals with OW/OB.

Current study also provides the first indication that ghrelin proteolysis increases in plasma after food intake in lean individuals, in a sex-independent manner, allowing to hypothesize that increased postprandial ghrelin proteolysis in lean individuals contributes to decrease plasma ghrelin levels after food intake. Since glycemia is well-known to transiently increase after food intake, it is possible that the postprandial increment of ghrelin proteolysis is also linked to glucose levels. In line with this possibility, a previous study found that a glucose load potently decreases ghrelin levels in lean individuals [31]. Further studies assessing blood samples at earlier time points will be required to test the extent to which postprandial increments of glycemia and ghrelin proteolysis correlate. Interestingly, ghrelin proteolysis did not increase after food intake in plasma of individuals with OW/OB, in which plasma ghrelin level shows a smaller decrease after meal intake, as previously shown [5] and confirmed here. Thus, it is likely that the lack of postprandial increase in ghrelin proteolysis in obesity contributes to impair the postprandial reduction of ghrelin levels, an alteration that has been proposed to reduce satiety feelings in individuals with obesity [47-49].

Although informative, this study is not without limitations. For instance, some aspects of our experimental design, such as the postprandial blood sample collection time or the sample size, were based on previous studies investigating the postprandial decline in ghrelin level [5,32,35-38]. However, such experimental design may have prevented us from detecting the postprandial change of ghrelin proteolytic activity in individuals with OW/OB, if an increase in such enzymatic activity takes place with a different kinetics or if it requires a higher sample size to be unmasked. Also, the time chosen to collect the postprandial blood sample did not allow us to detect the transient meal-induced increase of glycemia and, consequently, we could not evaluate if postprandial hyperglycaemia either was

differentially affected in individuals with OW/OB or correlated the postprandial increase of ghrelin proteolytic activity in lean individuals. The study did not include "sham-feeding" control groups and, consequently, a postprandial increase of ghrelin proteolytic activity may be related to its physiological diurnal rhythm. Despite these limitations, the current study was very valuable to gain insights about a novel aspect of the biology of ghrelin in human plasma.

Ghrelin des-acylation in plasma is fast and affects the circulating ghrelin level [50]. Indeed, intravenous administration of ghrelin in humans results in a peak of ghrelin levels, at 15 min after injection, and is rapidly eliminated [11,40]. Ghrelin des-acylation seems to be the main mechanism mediating the reduction of plasma ghrelin elevation since the referred increase of ghrelin level is followed by a peak of DAG level which then disappears from plasma more slowly than ghrelin [11,40]. Here, we found that ghrelin des-acylation was unaffected in individuals with OW/OB or postprandially suggesting that des-acylation is not a key mechanism mediating the decrease of plasma ghrelin level in those metabolic conditions. In line with our observations, it was reported that the level of serum butyrylcholinesterase activity, a ghrelin esterase in humans, was not altered during fasting [51]. Since our results indicate that ghrelin proteolysis is an important mechanism affecting the decrease of plasma ghrelin level in males with OW/OB, the mechanisms mediating the decrease of plasma ghrelin level in females with OW/OB remain uncertain. Given the fast rate of ghrelin des-acylation, it can be hypothesized that the observed decrease of plasma ghrelin level in the above referred conditions is due to a reduction of the secretion of ghrelin from the stomach [10], as it has been shown in obese mice [52]. Also, it is possible that des-acylation of circulating ghrelin is unaffected in plasma but regulated in some specific tissues, such as liver. Future studies in humans are needed to test these possibilities.

Conclusions

We provide here evidence indicating that ghrelin proteolysis in plasma increases in males with OW/OB and that it is acutely regulated by food intake in lean individuals. Also, we report that ghrelin des-acylation in plasma is not affected by body weight or food intake. Thus, current study helps to better understand the mechanisms controlling the changes of plasma ghrelin under different metabolic conditions in humans. Ghrelin proteolysis may be a putative pharmacological target to control circulating ghrelin level in humans. In particular, enhancement of ghrelin proteolysis in males with OW/OB or after food intake may help to further reduce plasma ghrelin level, with concomitant "beneficial"

effects such as appetite reduction and downregulation of mechanisms that increase glycemia.

Acknowledgements

We thank to all the participants of the study, to the deputy director of the Instituto Biológico (Dr. H. Giudicci), who kindly allowed the use of the facilities to perform the studies, to Carole Ribet-Travers, from Bertin Technologies that kindly provided the ELISA kits, to Spring Valdivia and Ana Luz Kruger for their technical assistance. This work was supported by grants from Fondo para la Investigación Científica y Tecnológica (FONCyT, PICT2017-3196, PICT2019-3054 and PICT2020-3270 to MP; PICT2019-256 to MFA; PICT2017-1216 to DL), CONICET (PUE105 to MP), The National Qatar Research Foundation (NPRP13S-0209-200315 to MP) and Fundación Florencio Fiorini (to MFA).

Conflict of interest

The authors declare no conflicts of interest. The sponsors had no role in the design of the study, the collection, analyses or interpretation of data, writing of the manuscript, or the decision to publish the results.

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Figure Legends.

Figure 1. Overview of methods to assess ghrelin des-acylation (A) and ghrelin proteolysis (B). See text for details. Eppendorf tube illustration adapted from scidraw.io (doi.org/10.5281/zenodo.6808872).

Figure 2. Preprandial plasma levels of ghrelin (A), DAG (B), ghrelin/DAG ratio (C), ghrelin des-acylation activity (D) and ghrelin proteolysis (E) in NW and OW/OB male participants. *, **, *** and **** indicate significant difference at $p<0.05$, $p<0.01$ $p<0.001$ $p<0.0001$ by Student´s t test or Mann Whitney test.

Figure 3. Correlations of ghrelin proteolysis with ghrelin (A), DAG (B), BMI (C), and glycemia (D) in male participants in preprandial conditions.

Figure 4. Preprandial and postprandial plasma levels of ghrelin (A), DAG (B), ghrelin des-acylation activity (C) and ghrelin proteolysis (D) in males with NW or OW/OB. Coloured points and lines show the geometric mean and each grey line represents the change within an individual participant. Exact P values are shown. Two-way repeated measures analysis of variance (ANOVA) with Bonferroni multiple-comparisons test was performed. *: significant difference vs. same group but different feeding condition. #: significant difference vs. different group but same feeding condition.

Figure 5. Preprandial and postprandial plasma levels of ghrelin (A), DAG (B) and ghrelin proteolysis (C) in females with NW or OW/OB. Coloured points and lines show the geometric mean and each grey line represents the change within an individual participant. Exact P values are shown. Two-way repeated measures analysis of variance (ANOVA) with Bonferroni multiple-comparisons test was performed. *: significant difference vs. same group but different feeding condition. #: significant difference vs. different group but same feeding condition.

Table 1: Baseline features of male participants

	Participants with NW	Participants with OW/OB	p-value
Age	34.7 ± 9.1	37.1 ± 5.0	N.S.
BMI	23.6 ± 0.9	30.9 ± 4.6	0.0001
Glycemia (g L^{-1})	0.99 ± 0.04	1.08 ± 0.07	0.0042
Insulin (µIU mL^{-1})	6.29 ± 3.21	14.28 ± 8.35	0.0139
HOMA index	1.55 ± 0.81	3.83 ± 2.31	0.0114

Abbreviations: BMI: body mass index; HOMA: homeostatic model assessment.

Data are presented as mean ± SD. Data analysis performed by t test.

Table 2: Baseline features of female participants

	Participants with NW	Participants with OW/OB	p-value
Age	34.5 ± 8.1	30.3 ± 7.9	N.S.
BMI	22.3 ± 2.1	29.1 ± 2.4	<0.0001
Glycemia (g L^{-1})	0.96 ± 0.05	0.98 ± 0.04	N.S.
Insulin (µIU mL^{-1})	5.15 ± 1.41	12.87 ± 4.42	<0.0001
HOMA index	1.22 ± 0.35	3.13 ± 1.11	<0.0001
Ghrelin (fmol mL^{-1})	40.26 ± 20.56	19.47 ± 9.60	0.0180
DAG (fmol mL^{-1})	63.77 ± 27.16	24.45 ± 9.27	0.0011
Ghrelin/DAG ratio	0.61 ± 0.21	0.77 ± 0.22	N.S.
Proteolytic activity (r.u.)	0.04 (0.02; 0.10)	0.06 (0.03; 0.21)	N.S.
Desacylase activity (r.u.)	1.31 ± 0.77	0.94 ± 0.12	N.S.

Abbreviations: BMI: body mass index; HOMA: homeostatic model assessment; DAG: desacyl-ghrelin.

Data are presented as mean ± SD and analysed by t-test for all parameters except for proteolytic activity, which is presented as median (IQR) and analysed by Mann Whitney test.

Credit author statement

Conceptualization, formal analysis, original draft preparation, supervision, overall responsibility for the project: MFA, MP.

Methodology, validation, formal analysis, investigation, resources and data curation; ASF, DC, DL, CS, PNDF, CRT, TL, LGL, SC, JAF, MFA, MP.

Funding acquisition, formal analysis, reviewing and editing, supervision: MFA, MP.

All authors approved the submitted and published versions.

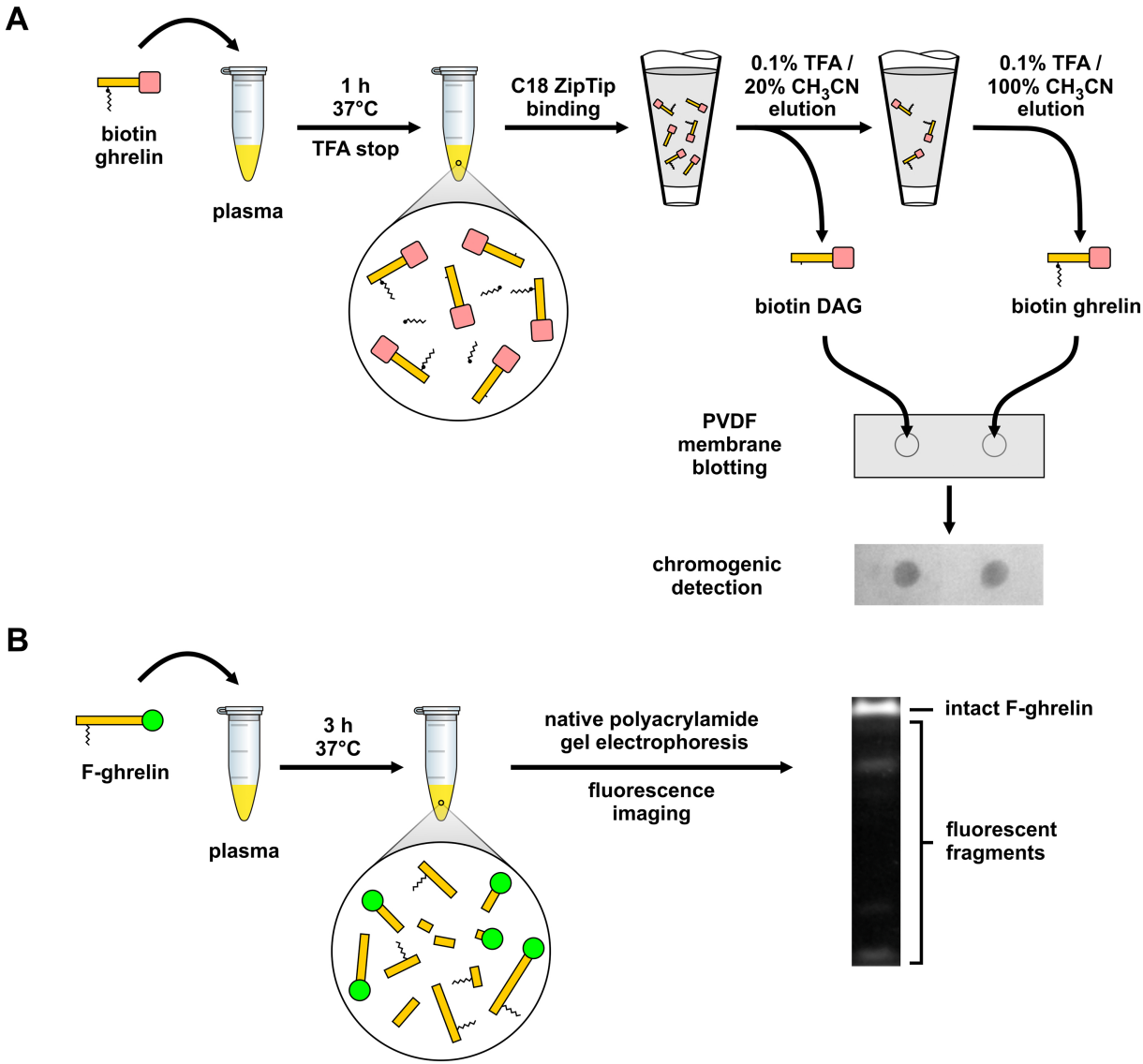
A
biotin ghrelin
plasma
1 h
37°C
TFA stop
C18 ZipTip binding
0.1% TFA / 20% CH3CN elution
0.1% TFA / 100% CH3CN elution
biotin DAG
biotin ghrelin
PVDF membrane blotting
chromogenic detection
B
F-ghrelin
plasma
3 h
37°C
native polyacrylamide gel electrophoresis
fluorescence imaging
intact F-ghrelin
fluorescent fragments

Figure 1

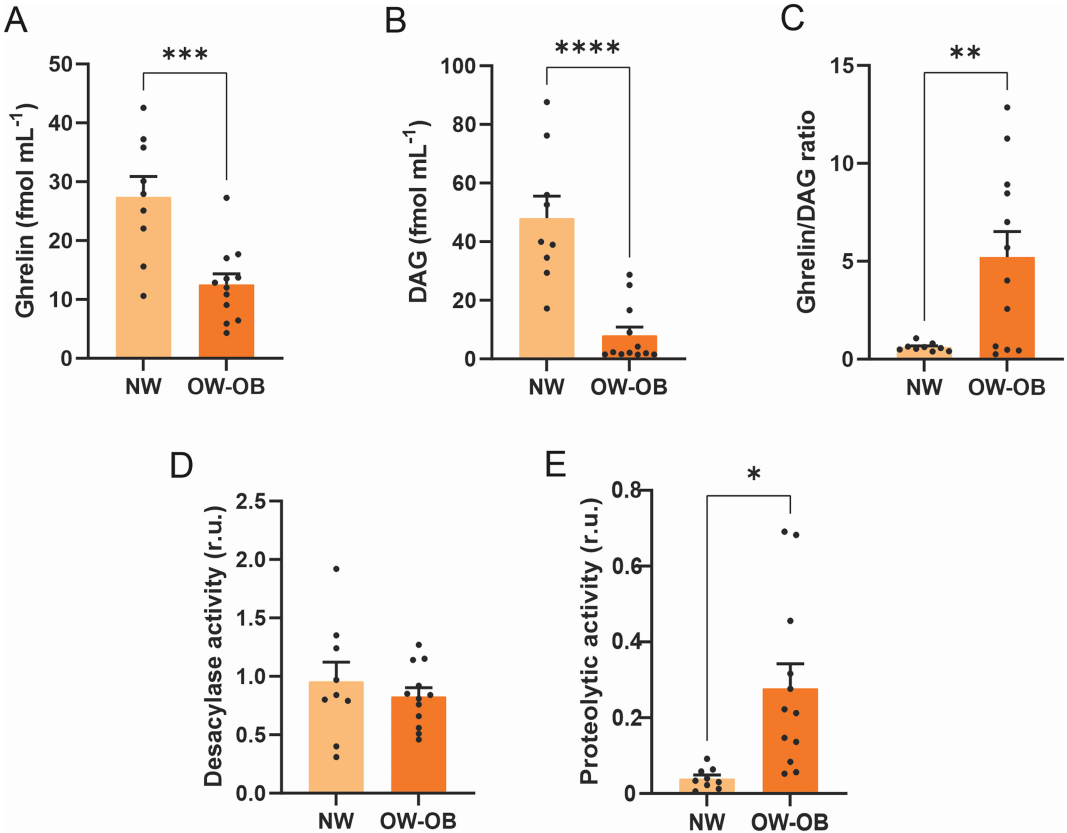
A
***
Ghrelin (fmol mL-1)
50
40
30
20
10
0
NW
OW-OB
B
****
DAG (fmol mL-1)
100
80
60
40
20
0
NW
OW-OB
C
**
Ghrelin/DAG ratio
15
10
5
0
NW
OW-OB
D
Desacylase activity (r.u.)
2.5
2.0
1.5
1.0
0.5
0.0
NW
OW-OB
E
*
Proteolytic activity (r.u.)
0.8
0.6
0.4
0.2
0
NW
OW-OB

Figure 2

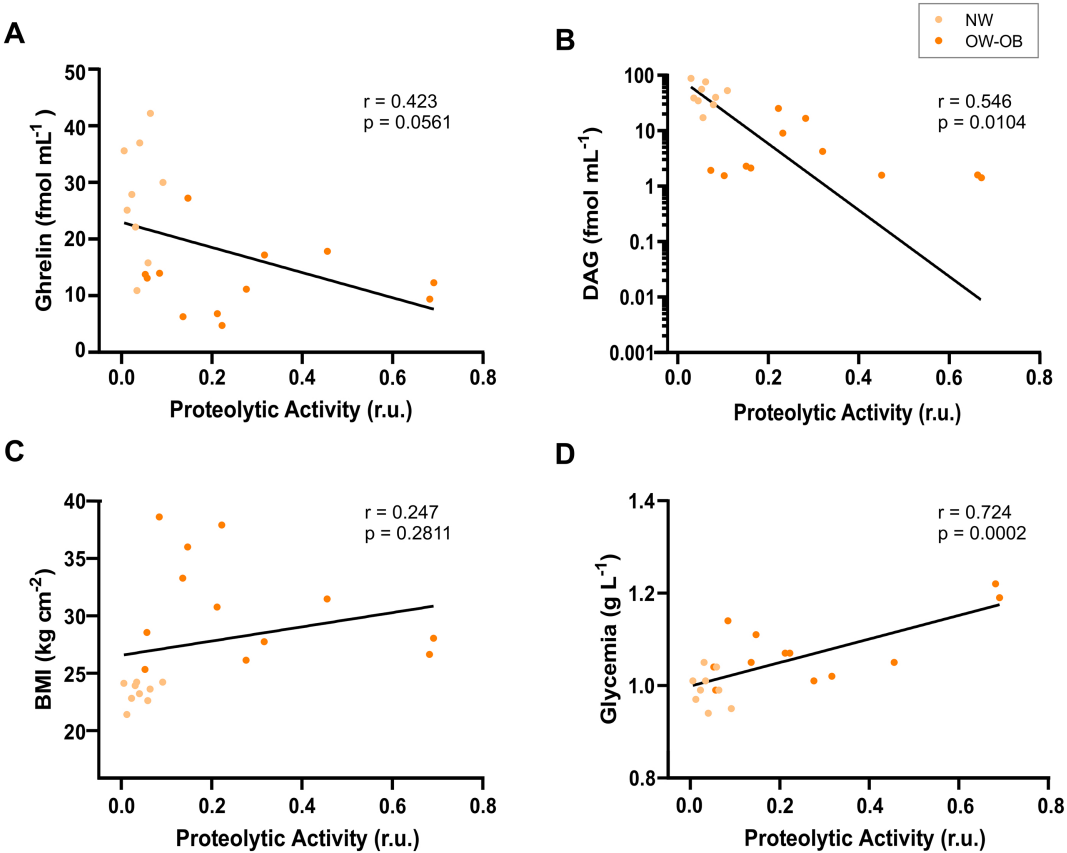
A
Ghrelin (fmol mL-1)
r = 0.423
p = 0.0561
Proteolytic Activity (r.u.)
B
NW
OW-OB
DAG (fmol mL-1)
r = 0.546
p = 0.0104
Proteolytic Activity (r.u.)
C
BMI (kg cm-2)
r = 0.247
p = 0.2811
Proteolytic Activity (r.u.)
D
Glycemia (g L-1)
r = 0.724
p = 0.0002
Proteolytic Activity (r.u.)

Figure 3

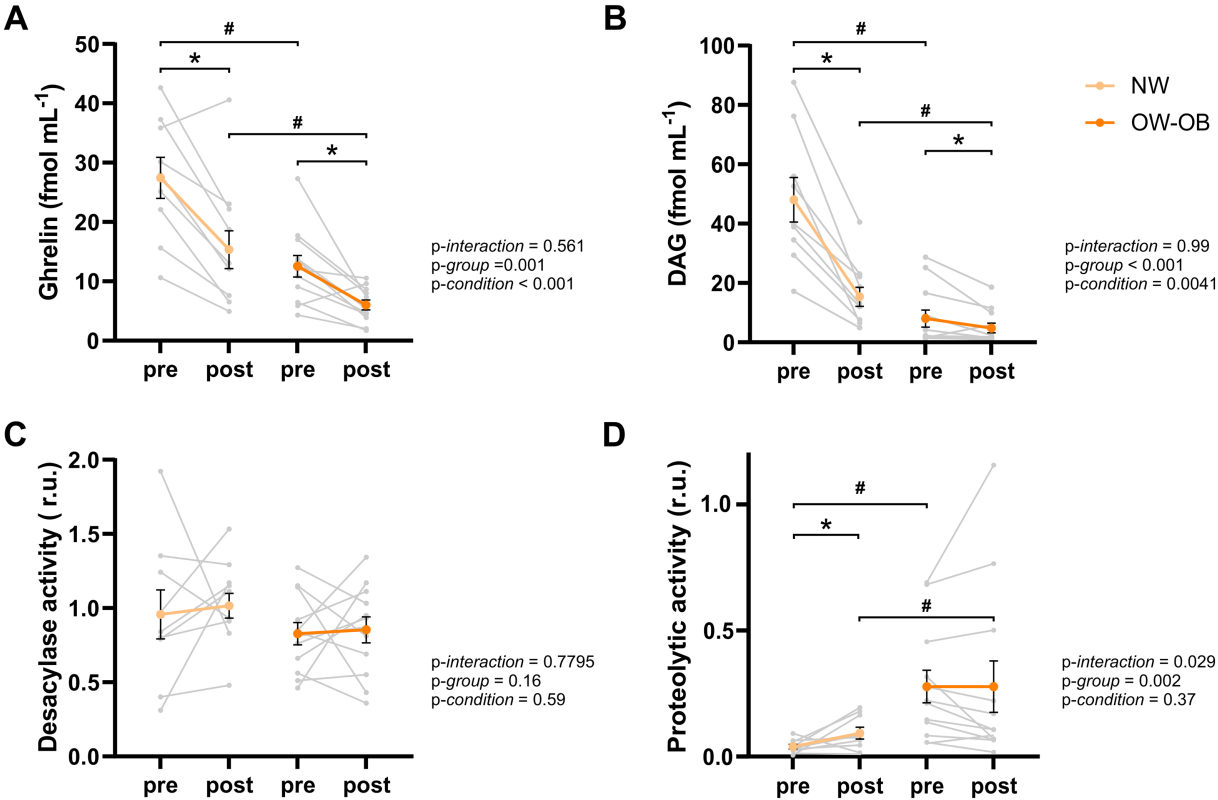
A
Ghrelin (fmol mL-1)
50
40
30
20
10
0
#
*
#
*
pre
post
pre
post
p-interaction = 0.561
p-group =0.001
p-condition < 0.001
B
DAG (fmol mL-1)
100
80
60
40
20
0
#
*
#
*
pre
post
pre
post
NW
OW-OB
p-interaction = 0.99
p-group < 0.001
p-condition = 0.0041
C
Desacylase activity (r.u.)
2.0
1.5
1.0
0.5
0.0
pre
post
pre
post
p-interaction = 0.7795
p-group = 0.16
p-condition = 0.59
D
Proteolytic activity (r.u.)
1.0
0.5
0.0
#
*
#
pre
post
pre
post
p-interaction = 0.029
p-group = 0.002
p-condition = 0.37

Figure 4

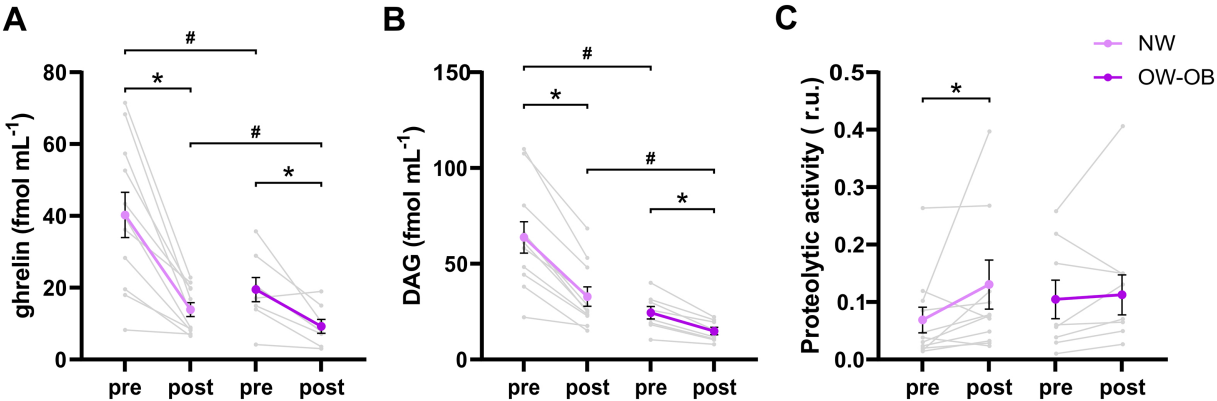
A
ghrelin (fmol mL-1)
80
60
40
20
0
#
*
#
*
pre
post
pre
post
p-interaction = 0.265
p-group = 0.037
p-condition < 0.001
B
DAG (fmol mL-1)
150
100
50
0
#
*
#
*
pre
post
pre
post
p-interaction = 0.059
p-group = 0.001
p-condition < 0.001
C
NW
OW-OB
Proteolytic activity (r.u.)
0.5
0.4
0.3
0.2
0.1
0.0
*
pre
post
pre
post
p-interaction = 0.597
p-group = 0.478
p-condition = 0.008

Figure 5