

## Simultaneous Decrease of Plasma Obestatin and Ghrelin Levels after a High-Carbohydrate Breakfast in Healthy Women

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### Summary

Ghrelin is a gut peptide produced mainly by stomach, well known to induce appetite stimulatory actions. Obestatin, a recently identified peptide derived from preproghrelin, was initially described to antagonize stimulatory effect of ghrelin on food intake. The postprandial response of obestatin and its relationship with ghrelin in humans remains unknown. We therefore investigated the postprandial response of obestatin and total ghrelin, acyl and desacyl ghrelin and neuropeptide Y (NPY) to a high-carbohydrate breakfast (1 604 kJ) in eight healthy women (age: 24.2±0.82 years; BMI 21.6±0.61 kg/m<sup>2</sup>). Blood samples were collected before the meal, and 30, 60, 90, 120 and 150 min after the breakfast consumption. Postprandial plasma obestatin concentrations significantly decreased compared with preprandial levels as well as total ghrelin concentrations and reached the lowest values 90 and 120 min after the meal consumption, respectively ( $p<0.05$ ). Plasma acyl and desacyl ghrelin concentrations decreased after the breakfast and reached lowest values in 30 and 60 min, respectively ( $p<0.05$ ). Plasma NPY concentrations were lower than preprandial levels 90 and 150 min after consuming breakfast ( $p<0.05$ ). In conclusion, we demonstrated in healthy young women that plasma obestatin concentrations decrease similarly to ghrelin after a high-carbohydrate breakfast.

### Key words

Obestatin • Total ghrelin • Acyl ghrelin • Desacyl ghrelin • Carbohydrate breakfast

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### Introduction

Ghrelin is a 28-amino acid peptide predominantly produced by the stomach, although its expression has also been confirmed in many other tissues (Kojima *et al.* 1999, Muccioli *et al.* 2002, Broglio *et al.* 2003, Bilgin *et al.* 2007). Ghrelin has been discovered as a natural ligand of the orphan growth hormone secretagogue (GHS) receptor (GHS-R) type 1a (GHS-R1a), which is widely distributed in both central and peripheral tissues (Kojima *et al.* 2001, Muccioli *et al.* 2002, Gnanapavan *et al.* 2002). Ghrelin secretion is mainly under metabolic control as it is increased by fasting and energy restriction but decreased by food intake, glucose and insulin (Lucidi *et al.* 2002, Shiiya *et al.* 2002, Ukkola 2003, Broglio *et al.* 2003, Nedvídková *et al.* 2003, Flanagan *et al.* 2003, Dostálová *et al.* 2007). In agreement with the major influence of nutrition, ghrelin levels are increased in anorexia and cachexia but reduced in obesity (Muccioli *et al.* 2002, Ukkola 2003, Rosická *et al.* 2003, Broglio *et al.* 2003, Dostálová and Haluzík 2007). Ghrelin is the first natural hormone known to date in which the hydroxyl group of one of its serine residues is acylated by n-octanoic acid (Kojima *et al.* 1999). This acylation is essential for binding to the GHS-R1a receptor, for the GH-releasing capacity of ghrelin and likely also for its other endocrine and physiological actions (Kojima *et al.* 1999, Matsumoto *et al.* 2001, Muccioli *et al.* 2001). However, unacylated ghrelin is not biologically inactive, as acylated ghrelin it exhibits some cardiovascular actions and the ability to modulate cell proliferation (Cassoni *et al.* 2001, Baldanzi

*et al.* 2002, Bedendi *et al.* 2003) and plays a role in the regulation of food intake (Asakawa *et al.* 2005). In this context, it is not by chance that unacylated ghrelin circulates at 2.5-fold higher concentration than the acylated form (Kojima *et al.* 2001, Muccioli *et al.* 2002).

Ghrelin-associated peptide obestatin is a recently discovered 23-amino acid peptide hormone derived from the same gene as ghrelin, first described by Zhang *et al.* (2005) to activate the orphan G protein-coupled receptor GPR39. However, several other groups failed to obtain reproducible obestatin binding and signaling in GPR39 receptor transfected cells (Jackson *et al.* 2006, Holst *et al.* 2007, Tremblay *et al.* 2007) even using the same conditions as previously reported by Zhang *et al.* (2005). Since the discovery of obestatin and its suppressive effect on food intake in fasted/refed mice (Zhang *et al.* 2005), the vast majority of subsequent studies (Gourcerol *et al.* 2006, Samson *et al.* 2007, Holst *et al.* 2007, Nogueiras *et al.* 2007, Yamamoto *et al.* 2007, Zizzari *et al.* 2007), except two (Tremblay *et al.* 2007, Carlini *et al.* 2007), could not reproduce the initially reported anorexigenic property of obestatin. In addition to the recent controversy over the effects of obestatin, further studies researching peripheral or central injection of obestatin in mice found that it does not affect food intake (Gourcerol *et al.* 2006, De Smet *et al.* 2007, Bassil *et al.* 2007, Nogueiras *et al.* 2007). The role of human obestatin and the kind of immunoreactivity measured in human has still been highly disputable (Bang *et al.* 2007, Chartrel *et al.* 2007) and the interaction between obestatin and ghrelin in the regulation of food intake has also been under question (Seoane *et al.* 2006, Nogueiras *et al.* 2007). However, the circulating preprandial ghrelin to obestatin-like immunoreactivity ratio may be of importance in the etiology and pathophysiology of obesity (Guo *et al.* 2007).

The identification of obestatin as novel peptide hormone derived from the same gene as ghrelin has recently added further complexity to the ghrelin physiology. Despite rapid progress in this field of interest, many questions still remain to be answered, including the regulation of ghrelin and obestatin secretion and their precise physiological endocrine roles. To our best knowledge, no data have been published yet regarding the effect of high-carbohydrate breakfast consumption on postprandial plasma obestatin and total, acyl and desacyl ghrelin response in healthy weight women. Thus, the aim of our study was to characterize plasma obestatin and ghrelin responses to a high-

carbohydrate breakfast in healthy women and to investigate the physiological relationship of these hormones with insulin and neuropeptide Y (NPY).

## Methods

### Subjects

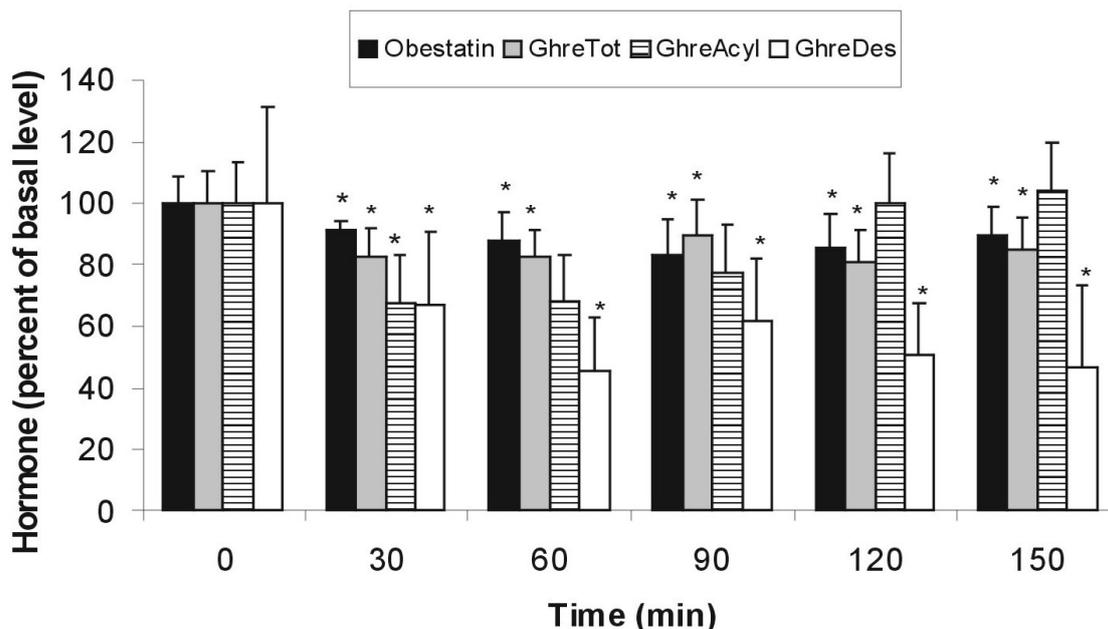
This study was performed in accordance with the Declaration of Helsinki and was approved by the Ethic Review Committee of the Institute of Endocrinology in Prague. Prior to the study, all participants provided written informed consent concerning participation in the study.

Eight healthy Czech women (age: 24.2±0.82 years; body mass index (BMI) 21.6±0.61 kg/m<sup>2</sup>) were enrolled in this study. The women had no history of eating disorders, had normal electrocardiogram (ECG), blood count, liver and renal function. All women had regular menstrual cycles and were in the follicular phase of their cycle at the time of the study. Women were recommended to avoid vigorous physical activity for 24 hours prior to the experiment, consumed a standardized dinner at 6 pm the day before the experiment and were asked not to eat and drink during the night. All participants were admitted to the Institute of Endocrinology at 7.30 am. Firstly, all of them underwent physical and ECG examination, and body weight and height measurements. The study took place in a room kept at 23-25 °C and the women were lying in bed during study. Overall, the study lasted about 3.5 hours and the protocol consisted of high-carbohydrate breakfast consumption and blood withdrawals.

### Study design

Each subject received a high-carbohydrate breakfast with a total energy content of 1604 kJ, consisting of 81.9 g carbohydrates, 8.8 g protein and 3.4 g fat in the form of a white bread roll (90 g) and strawberry jam (50 g). In addition, the subjects consumed 250 ml of fruit tea without sugar or other sweetener with the meal. Participants were given 15 minutes to consume their meal.

Blood samples were drawn from the cubital vein using an intravenous cannula, the first blood drawn was collected before meal, and then 30, 60, 90, 120 and 150 min after breakfast consumption. Blood samples were collected into chilled polypropylene tubes containing Na<sub>2</sub>EDTA and antilysin. Plasma was immediately separated by 15-min centrifugation at 5 °C and stored at



**Fig. 1.** The percent of postprandial decrease in plasma levels of obestatin, total ghrelin, acyl and desacyl ghrelin. GhreTot - Ghrelin total, GhreAcyl - Ghrelin Acyl, GhreDes - Ghrelin desacyl. \*change in hormone level compared with preprandial status (time 0'),  $p < 0.05$

-70 °C until being assayed. Plasma for acyl and desacyl ghrelin assay was acidified by 1N HCl in ratio 1/10 after centrifugation.

#### Analytical measurements

Plasma obestatin immunoreactivity was measured with a commercial RIA kit (Phoenix Pharmaceuticals Inc., Belmont, CA, U.S.A.), the intra- and interassay variability was 5.0 % and 14.2 %, respectively, sensitivity was 50 pg/ml. Total plasma ghrelin, leptin and NPY were determined using commercially available RIA kits (Linco Research, Inc., St. Charles, Missouri, U.S.A.). The intra- and interassay of total ghrelin was 6.4 % and 16.3 %, sensitivity 93 pg/ml; intra- and interassay of leptin was 4.9 % and 4.5 %, sensitivity 0.5 ng/ml; intra- and interassay of NPY was 3.3 % and 11.6 %, sensitivity 6 pmol/l. Acyl and desacyl plasma ghrelin were determined by ELISA kit (Linco Research, Inc., St. Charles, Missouri, U.S.A.). The intra- and interassay of acyl ghrelin was 4.3 % and 3.5 %, sensitivity 2.5 fmol/ml; the intra and interassay of desacyl ghrelin was 3.5 % and 6.7 %, sensitivity 12.5 fmol/ml. Plasma insulin levels were determined by commercial RIA kit (Immunotech, Inc., Prague, Czech Republic), the intra- and interassay of insulin was 3.3 % and 2.9 %, sensitivity 0.5  $\mu$ IU/ml.

#### Statistical analysis

All results are expressed as means  $\pm$  SEM. The

time courses of dependent variables were evaluated by repeated measures ANOVA model. The differences between subgroups were evaluated using least significant difference multiple comparisons. The statistical significance  $p < 0.05$  was chosen for both ANOVA testing and multiple comparisons. Due to non-Gaussian data distribution in all dependent variables, the dependent variables underwent power transformations to attain distributional symmetry and a constant variance in the data as well as in residuals. The statistical software NCSS 2002 (Kaysville, UT, USA) was used for data analysis.

## Results

The postprandial responses of the studied hormones are summarized in Table 1. Postprandial plasma obestatin and total ghrelin concentrations significantly decreased compared with preprandial levels and reached their lowest values 90 and 120 min after meal consumption, respectively ( $80.9 \pm 4.89$  % and  $80.7 \pm 4.33$  %, respectively,  $p < 0.05$ ). Plasma acyl ghrelin concentrations decreased significantly only in 30. min after the meal ( $67.7 \pm 13.67$  %,  $p < 0.05$ ). Plasma desacyl ghrelin concentrations decreased significantly after the breakfast and reached the lowest value 60 min after breakfast ( $45.3 \pm 11.88$  %,  $p < 0.05$ ) (Fig. 1). Preprandial ghrelin/obestatin ratio was  $5.9 \pm 0.5$  and there was no significant change in this ratio during the postprandial period. Plasma insulin concentrations increased

**Table 1.** Pre- (0 min) and postprandial (30, 60, 90, 150 min) plasma levels of the studied hormones.

|                           | 0 min      | 30 min     | 60 min     | 90 min     | 120 min    | 150 min    |
|---------------------------|------------|------------|------------|------------|------------|------------|
| <i>Obestatin</i> (pg/ml)  | 181±15.3   | 165±4.76*  | 159±14.8*  | 150±17.4*  | 155±16.9*  | 162±14.7*  |
| <i>GhreTot</i> (pg/ml)    | 1053±106   | 868±83*    | 865±80.9*  | 942±110*   | 850±90.4*  | 899±88.1*  |
| <i>GhreAcyl</i> (fmol/ml) | 11.61±1.54 | 7.86±1.2*  | 7.96±1.2   | 8.95±1.42  | 11.6±1.94  | 12.1±1.86  |
| <i>GhreDes</i> (fmol/ml)  | 95.9±29.5  | 64.3±15.3* | 43.4±7.63* | 59.2±11.7* | 49.0±8.19* | 44.6±11.9* |
| <i>Ghre/Obest</i>         | 5.88±0,50  | 5.35±0,33  | 5.52±0,32  | 6.58±0,95  | 5.58±0,51  | 5.60±0,35  |
| <i>Insulin</i> (μIU/ml)   | 5.71±0.75  | 37.8±5.02* | 35.4±2.45* | 28.9±2.94* | 30.4±4.32* | 16.7±2.82* |
| <i>NPY</i> (pmol/l)       | 47.5±7.54  | -          | 42.4±7.26  | 36.9±6.93* | -          | 36.7±19.6* |

GhreTot - Ghrelin total, GhreAcyl - Ghrelin acyl, GhreDes - Ghrelin desacyl, Ghre/Obest - Ghrelin/Obestatin ratio, NPY – neuropeptide Y  
\*change in hormone level compared with preprandial status (time 0'),  $p < 0.05$

**Table 2.** Relationship of obestatin, total ghrelin, acyl and desacyl ghrelin with other measured biochemical and hormonal parameters calculated with all six times (0, 30, 60, 90, 120, 150 min.) included.

|                  |   | Obestatin     | GhreTot      | GhreAc       | GhreDes      | Ghr/Ob        | Insulin | NPY          | BMI          |
|------------------|---|---------------|--------------|--------------|--------------|---------------|---------|--------------|--------------|
| <i>Obestatin</i> | r | -             | <b>0.661</b> | 0.284        | <b>0.460</b> | <b>-0.309</b> | -0.143  | <b>0.805</b> | -0.197       |
|                  | p | -             | <b>0.000</b> | 0.068        | <b>0.002</b> | <b>0.047</b>  | 0.366   | <b>0.000</b> | 0.210        |
| <i>GhreTot</i>   | r | <b>0.661</b>  | -            | <b>0.363</b> | <b>0.666</b> | <b>0.414</b>  | -0.190  | <b>0.490</b> | -0.048       |
|                  | p | <b>0.000</b>  | -            | <b>0.018</b> | <b>0.000</b> | <b>0.006</b>  | 0.228   | <b>0.009</b> | 0.762        |
| <i>GhreAcyl</i>  | r | 0.284         | <b>0.363</b> | -            | 0.152        | 0.033         | -0.208  | <b>0.449</b> | -0.266       |
|                  | p | 0.068         | <b>0.018</b> | -            | 0.336        | 0.836         | 0.187   | <b>0.019</b> | 0.089        |
| <i>GhreDes</i>   | r | <b>0.460</b>  | <b>0.666</b> | 0.152        | -            | <b>0.357</b>  | -0.105  | <b>0.459</b> | 0.093        |
|                  | p | <b>0.002</b>  | <b>0.000</b> | 0.336        | -            | <b>0.020</b>  | 0.506   | <b>0.016</b> | 0.557        |
| <i>Ghre/Ob</i>   | r | <b>-0.309</b> | <b>0.414</b> | 0.033        | <b>0.357</b> | -             | -0.100  | -0.303       | <b>0.408</b> |
|                  | p | <b>0.047</b>  | <b>0.006</b> | 0.836        | <b>0.020</b> | -             | 0.530   | 0.124        | <b>0.007</b> |

GhreTot – Ghrelin total, GhreAcyl – Ghrelin Acyl, GhreDes - Ghrelin desacyl, Ghre/Obest – Ghrelin/Obestatin ratio, NPY – neuropeptide Y, BMI – Body Mass Index, r = correlation coefficient, p = P value (p values <0.05 are in bold)

significantly after the meal with the highest value in 30 min after the meal ( $p < 0.05$ ). Plasma concentrations of NPY were significantly lower than basal levels in 90 and 150 min after the meal ( $p < 0.05$ ).

The relationship of obestatin with the other studied parameters is shown in Table 2. Obestatin was positively related to total ghrelin, desacyl ghrelin and NPY. Total, acyl and desacyl ghrelin correlated positively to each other and also with NPY. Ghrelin/obestatin ratio was positively related to BMI.

## Discussion

The present study was designed to investigate

the effect of a high-carbohydrate breakfast consumption on total, acyl and desacyl ghrelin and ghrelin-associated peptide obestatin in healthy normal-weight women. We demonstrated for the first time that plasma obestatin levels significantly decrease after consumption of a high-carbohydrate breakfast in healthy women in a similar fashion as do total, acyl and desacyl ghrelin. The positive relationship of obestatin with total ghrelin in the postprandial period indicates that these two cleavage products of one gene could act in a similar fashion to increase food intake in healthy humans. This idea is further confirmed by the positive correlation between obestatin and orexigen NPY. However, the relationship of obestatin with the desacyl form of ghrelin does not seem

to play with the idea of an orexigenic effect of obestatin (Asakawa *et al.* 2005).

The initially claimed anorexigenic role of obestatin in mice was highly negated by almost all of subsequently published studies (Gourcerol *et al.* 2006, Samson *et al.* 2006, Holst *et al.* 2007, Nogueiras *et al.* 2007, Yamamoto *et al.* 2007, Zizzari *et al.* 2007). No change or a modest reduction (20 %) in plasma obestatin levels has been reported between non-fasted and fasted state in mice (Zhang *et al.* 2005, Zizzari *et al.* 2007). The interaction between obestatin and ghrelin in the regulation of food intake has also been under question. Indeed, while the initial report found that obestatin could reverse the ghrelin-induced stimulation of food intake in fasted mice, such interaction was not confirmed subsequently upon an acute obestatin injection in fed or fasted rats (Seoane *et al.* 2006) or during the 7-day treatment with obestatin in mice (Nogueiras *et al.* 2007). Here we show that obestatin exhibits, similarly to ghrelin, postprandial reduction after a high-carbohydrate meal in healthy women, suggesting rather an additive role of these two peptides in postprandial satiation. Furthermore, we negate previous observation of rapid degradation of circulating obestatin as well as no effect of food intake on circulating obestatin levels.

Regarding human studies, the circulating preprandial ghrelin to obestatin ratio was elevated in human obesity, whereas two hours postprandially this ratio was unchanged in obese compared to lean (Guo *et al.* 2007), rising the hypothesis that the preprandial ghrelin to obestatin ratio may be of importance in the etiology and pathophysiology of obesity. We found the ghrelin/obestatin ratio to be positively related to BMI in healthy young women, whereas neither ghrelin or obestatin correlated to BMI. This might suggest an interesting role of balance between these two hormones as related to other hormonal, metabolic and anthropometric parameters and needs to be further elucidated. However, based on the data of Chartrel *et al.* (2007), the question about what is really measured in human studies is still unanswered together with the exact role of obestatin as an anorexigenic contraregulator to ghrelin or orexigenic hormone playing in a similar fashion to ghrelin. Bang *et al.* (2007) found no evidence for the existence of obestatin as an unique, endogenous peptide and the data of this study rather suggest that circulating and stored peptides derived from the carboxyl terminal of proghrelin (C-ghrelin) are consistent in length with proghrelin (29-94) and respond to metabolic

manipulation, at least in humans, in similar fashion to ghrelin (1-28). However, our group previously demonstrated that whatever we call immunoreactivity as measured by the Phoenix kit, this molecule is different than ghrelin and exhibits relationships with anthropometric parameters in cases where ghrelin fails to correlate (Hainer *et al.*, unpublished results). It is possible that in certain circumstances, i.e., starvation, the ghrelin gene is cleaved differentially than under physiological conditions. The ratio of ghrelin/obestatin and acyl/desacyl ghrelin may play a role in the regulation of food intake in humans. Another possible explanation of the simultaneous postprandial decrease of obestatin with ghrelin is that the function of obestatin may be to antagonize orexigenic ghrelin action after meal consumption, i.e., some kind of a negative feedback may exist between these two peptides. Our results pointed out the importance of measurement not only of total ghrelin, but also of its acyl and desacyl forms together with other ghrelin gene-derived peptides in order to better interpret the data.

Ghrelin is considered to be an upstream regulator of the orexigenic peptides NPY and AgRP (Rosická *et al.* 2002, Pfaff *et al.* 2004, Miura *et al.* 2006, Dardennes *et al.* 2007). Recent studies demonstrated a significant enhancement of plasma NPY levels after ghrelin injection in humans (Coiro *et al.* 2006) and stimulatory action of ghrelin on NPY gene transcription in vitro (Goto *et al.* 2006). Our results have shown a positive correlation between obestatin, total ghrelin, acyl ghrelin, desacyl ghrelin and NPY. This supports a role of proghrelin-derived peptides in the regulation of food intake and energy storage via NPY, although the mechanisms by which these peptides stimulates NPY neurons are not clear at all.

The postprandial decrease of total ghrelin after a high-carbohydrate breakfast in healthy individuals has been documented several times (Monteleone *et al.* 2003, Blom *et al.* 2005, Marzullo *et al.* 2006). However, what has not yet been established is the role of acyl and desacyl form of ghrelin in the postrandial regulation of satiety. Meal intake significantly suppressed acyl ghrelin by 38 % in healthy humans and serum insulin best predicted plasma acyl ghrelin concentrations accounting for 97 % of its variation (Lucidi *et al.* 2004). In agreement with this, a high-carbohydrate breakfast significantly decreased serum acyl ghrelin levels in healthy individuals (Tentolouris *et al.* 2004). In contrast to acylated ghrelin, desacyl ghrelin was shown to induce

a negative energy balance by decreasing food intake and delaying gastric emptying (Asakawa *et al.* 2005). Central desacyl ghrelin may activate orexin-expressing neurons in mice, perhaps functioning in feeding regulation (Toshinai *et al.* 2006). Our results have shown that not only total ghrelin, but also its acyl and desacyl form as well as obestatin significantly decrease after a high-carbohydrate breakfast. What is the role of the individual reduction of these hormones is highly speculative. One hypothesis which has been postulated is that unacylated ghrelin could be used to blunt the effects of acylated ghrelin (Broglio *et al.* 2004, Gauna *et al.* 2004) and that the ratio of acylated ghrelin and unacylated ghrelin production might help to regulate the balance between adipogenesis and lipolysis in response to nutritional status (Thompson *et al.* 2004). The type of relationship that may exist between ghrelin and obestatin has not yet been established, but it is possible that postprandially,

obestatin may blunt the effect of ghrelin in healthy humans.

In conclusion, we demonstrated that in healthy women plasma obestatin concentrations decrease similarly to ghrelin after a high-carbohydrate breakfast. Further investigation is needed to classify the role of the cleavage products of the ghrelin gene in human physiology.

### Conflict of Interest

There is no conflict of interest.

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