Original Article

Pre- and postprandial acylated ghrelin in obese and normal weight men

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Background and Objectives: The prevalence of obesity in Indonesia has increasing. We have assessed the relationship between plasma acylated ghrelin hormone and self-reported appetite ratings (hunger and desire to eat) in obese and normal weight men. **Methods and Study Design:** Thirty-two men participated in an experimental study, where acylated ghrelin and self-reported appetite ratings were compared between a test group of 16 obese men and a control group of 16 men with normal body mass indices. The participants were administered isocaloric mixed meals, and measurements were taken at 0 (before eating), 30, 60, and 120 minutes postprandial. Data were analysed using an independent *t* test, the Mann–Whitney U test, the Pearson correlation, the Spearman rank-order correlation, trapezoidal rule analysis for the area under the curve, and receiver operating curve analysis to determine the optimal cut-off values, sensitivity, and specificity. **Result**s: Acylated ghrelin concentrations were higher in the test group than in the control group at all time points ($p<0.01$). There were no significant differences in the appetite ratings between the two groups at any time ($p>0.05$). There was no correlation between the acylated ghrelin concentration and appetite rating. According to the receiver operating curve analysis (sensitivity: 88%; specificity: 100%), the cut-offs for optimal acylated ghrelin immediately before eating and 30 minutes after eating, averaged 2332 pg/mL and 2710 pg/mL, respectively. **Conclusions:** The effect on obesity will depend on associated changes in deacylated ghrelin. Acylated ghrelin increases in obese individuals pre- and 30, 60, 90 and 120 minutes post prandial.

Key Words: acylated ghrelin, appetite, obesity, energy homeostasis, hunger

INTRODUCTION

Obesity, the excessive accumulation of body fat, is a risk factor for chronic diseases such as heart and blood vessel disease, diabetes mellitus, and cancer.[1] Initially, obesity attracted attention only in developed countries; however, its prevalence has begun to increase in numerous developing countries, including Indonesia. From 2007 to 2013, the percentages of obese men and women in Indonesia increased from 13.8% to 19.7% and 14.8% to 32.9%, respectively.[2] This trend will continue if preventative measures are not implemented.

Obesity results from a positive energy balance or energy intake exceeding energy expenditure. The human brain has a key role in regulating energy homeostasis. For example, the endocrine system stimulates hunger and the desire to eat.[3] One of the hormones that regulates energy homeostasis is ghrelin,[4] an endogenous gastric peptide hormone synthesised by the endocrine X/A-like cells of the fundus mucosa and expressed in many tissues, such as the duodenum and jejunum.[5] Its production involves the transcriptional, translational, and posttranslational modification of the ghrelin gene. The final products are two forms of the hormone: deacylated ghrelin (DAG) and acylated ghrelin (AG). The enzyme responsible for catalysing AG synthesis is ghrelin O-acyltransferase (GOAT). DAG and AG have different functions in energy homeostatic processes.[6]

AG transmits its signal through the central and peripheral pathways and the vagus nerve. AG is locally synthesised by the hypothalamus, and, when secreted by the stomach, it reaches the brain through the blood-brain barrier. In the hypothalamus, AG activates the arcuate nucleus (ARC) and the paraventricular nucleus. Neurons expressing AG transmit signals to ARC neurons, and through a series of molecular mechanisms, hunger and the desire to eat are increased. DAG is an antagonist of AG.[7]

The AG hormone is currently a target of obesity therapy using the GOAT inhibitor.[8] This study was not only investigated AG levels and self-reported appetite ratings are compared between men classified as obese and non obese, but also assessed the optimal cut-off, sensitivity,

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and specificity of AG by using a diagnostic test. Participants were administered isocaloric mixed meals and measurements were taken at 0 (before eating) 30, 60, and 120 minutes after eating.

MATERIALS AND METHODS

Participants

The study sample was divided into two groups: 16 obese men (BMI ≥25.1)[9] and 16 normal weight men (BMI=18.5–22.9). Prescreening determined all participants to be healthy, with no previous history of hypercholesterolaemia, diabetes mellitus, or hypertension. The Committee of Ethics of the Medical Faculty of Brawijaya University approved this research (no. 389/EC/KEPK/07/2015).

Test meal

Test meals were taken from popular fast food restaurants located in Malang city that are frequently choosen by the community. The meal have comparable amount of energy (500–600 kcal/ serving). Every respondent from both group are served 1 test meal for their breakfast. The nutrient composition of the meals were analysed using a bomb calorimeter for energy analysis, Kjedahl method for protein, soxhlet for fat anlysis and enzymatic analysis for fibre. The composition of test meals is described in Table 2.

Study design

This study used a true experimental design, where the two groups were compared in terms of pre- and postprandial plasma AG and self-reported appetite ratings. The last meal was consumed at 21:00 that contained 25% of total daily energy expenditure. In the morning, fasting subject (minimum of 10 hours) arrived for meal-test day. Fasting blood sample and writing appetite ratting were conducted before breakfast. Thus subject consumed 1 fast food for around 25 minutes and water are provided. Subsequent blood sampling and complete appetite rating were conducted at 30th, 60th and 120th minutes after breakfast.

Blood sample and biochemical analysis

Blood plasma samples were centrifuged at 3000 rpm at 25°C for 10 minutes using a tabletop centrifuge (PLC-05 model, Gemmy Industrial Corp., Taipei, Taiwan, associated with Cannic Inc., USA). To test the blood samples for AG, a sandwich ELISA (Elabscience, Biotechnology, Beijing) was performed. A kit with the catalogue number E-EL-H2002 was used. Plasma samples were placed in a microtiter plate containing a specific antibody (A-GHRL) and incubated at 37°C for 90 minutes. Then, a specific biotinylated detection antibody and streptavidin horseradish peroxidase conjugate were added to each microplate and incubated at 37°C for 90 minutes. Substrate and stop solutions were added in a dark room until a colour change occurred. Optical density (OD) was determined using the ELISA reader with the wavelength of 450±2 nm. The concentration of the sample hormone was calculated by comparing the OD value with the standard value.

Appetite ratings

The Visual Analog Scale (VAS) was used to measure appetite ratings.[10] The participants were requested to fill out VAS hunger (Q1) and desire to eat (Q3) forms at 0 minutes (before eating) and 30, 60, and 120 minutes after eating. The VAS forms were then oriented on a 100-mm vertical line.

Statistical analysis

The data are presented as the mean±standard deviation (SD) per time of each group. The collected data were raw AG, raw appetite ratings, $AUC_{0\text{-}120}$ AG, and $AUC_{0\text{-}120}$ appetite ratings, where $AUC_{0\text{-}120}$ was obtained using the area under the curve (AUC) method by calculating the size of the graphic trapezoid. The collected data were then subjected to a normality test. The collected data were then subjected to a normality test. If the distribution was normal, an independent t test and Pearson correlation test were used; however, if the distribution was not normal, the Mann–Whitney U test and Spearman correlation test were applied. A receiver operating characteristic (ROC) curve analysis of the raw AG data indicated cut-offs, and the diagnostic test was interpreted using the 2×2 approach to determine the sensitivity and specificity. The formula $a/(a + c)\times100\%$ was used for sensitivity, and the formula $b/(b + d)\times100\%$ was used for specificity. Statistical analysis was conducted using SPSS Version 22 (IBM, Chicago, IL, USA).

RESULTS

Characteristics of the research participants

This study's sample consisted of 32 men between the ages of 19 and 29 years. The participants were evenly divided into two groups: 16 with normal BMIs and 16 obese men. The participant characteristics are shown in Table 1. There were significant differences between the characteristics of the two groups in variables including body weight, BMI, percentage of body fat, percentage of waist fat, and waist and hip circumferences, which was the distinguishing characteristic of the two groups ($p<0.01$). There were also significant differences ($p<0.01$) in systolic blood pressure (SBP), diastolic blood pressure (DBP), total cholesterol content, and leptin content. Although the obese group had higher average SBP, DBP, and total cholesterol content, all indicators were classified as normal and not in the hypertension or hyperlipidaemia categories. There were no significant differences in age, body height, or random blood glucose content between the two groups.

Nutritional information of the fast food

The nutritional information of the fast food used in this research is shown in Table 2. The food ranging from 546 to 593 kcal were used. Although the foods had different energy contents, they were considered isocaloric if they were within the limit of 24%–28% of the total energy required by the participants breakfast. The total energy requirement was adjusted to the nutrient adequacy rate of Indonesian people.[11]

The corelation of appetite ratings

The Hunger and the desire to eat corelation are presented in Figures 1. The results indicate a significant positive corelation between hunger score (VAS Q1) and desire to

Table 1. Characteristics of the research participants

Characteristic	Obese (Mean±SD)	Normal weight (Mean±SD)	*p* value
Age (year)	21.4±1.9	20.6±1.08	0.18
Weight (kg)	97.0±1.93	60.9±5.66	<0.01
Height (cm)	170±5.5	169±6.27	0.89
BMI (kg/m^2)	33.6±4.78	21.25±1.05	<0.01
% body fat	30.2±3.75	17.00±5.07	<0.01
% waist fat	16.7±4.14	4.68±1.01	<0.01
Waist circumference (cm)	105±12.0	75.43±4.20	<0.01
Flank circumference (cm)	114±8.69	93.5±11.9	<0.01
Systolic blood pressure (mmHg)	124±9.57	117±6.83	0.04
Diastolic blood pressure (mmHg)	80.6±4.4	75.0±5.16	<0.01
Random blood sugar (g/dL)	122±21.1	103±12.2	0.16
Cholesterol (g/dL)	135±27.7	94.8±49.8	<0.01
Leptin (pg/mL)	61.9±21.8	21.6±9.11	<0.01

Table 2. Characteristics of the fast food

Fast food characteristics	Range
Energy density (kcal/g)	1.66–3.88
Energy (kcal)	546–593
Serving size (g)	153–332
Fat (%)	2.41–6.85
Protein (%)	1.85–4.54
Carbohydrate (%)	10.2–19.3
Soluble fiber (g)	5.30–6.56

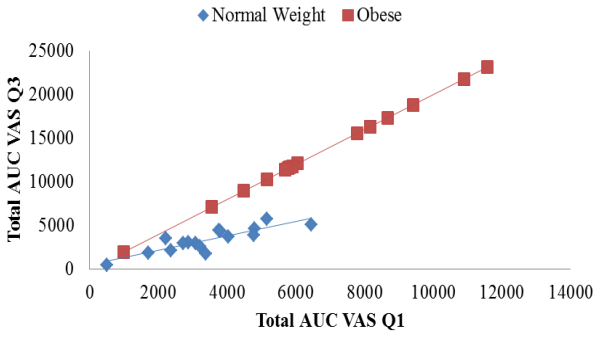


Figure 1. Significant correlations were found between appetite rating for hunger (AUC VAS Q1) and desire to eat (AUC VAS Q3)

eat (VAS Q3) for non obese (p<0.001; R=0.859) and obese (p<0.001; R=0.962). From Figure 1 revealed the corelation between hunger score and desire to eat was stronger on obese group. The data were also processed using the $(AUC)_{0\text{-}120}$; however, there were no significant differences in the two variables between the two groups (Table 3).

Acylated ghrelin

The average±SD of AG concentrations for the two groups measured before eating and 30, 60, and 120 minutes after eating is presented in Figure 2. The independent t test showed a significant difference between the two groups at each time point (p<0.01). However, the AG concentrations of both groups exhibited a similar graphical pattern: low at the 0th minute, an increase until the 30th minute, and then a slow decrease. One difference between the groups was observed between the 60th and 90th minutes, during which interval the control group experienced an increase in the AG concentration. The AG concentrations of the control group exhibited a circadian rhythm, with the AG increasing until the 30th minute and then decreasing until they were comparable to those measured prior to eating. In addition, the AG concentration was analysed using AUC_{0-120}; significant differences were found between the participant groups (p<0.01; Table 3).

Correlation between appetite ratings and AG hormonal content

In the control group, the Pearson correlation test revealed no significant difference between the appetite rating and AG hormonal content for all variables studied. However, the experimental group showed a relatively strong correlation between the desire to eat and AG before eating at the 30th minute and between hunger and AG at the 60th minute. The other time points exhibited a weak correlation (Table 4).

Acylated ghrelin diagnostic test as an alternative diagnosis for obesity

The cut-off AG concentration for each time point is listed in Table 5. The ROC method was used to determine the cut-off with a 2×2 table (Figure 3). The highest sensitivity was observed at 0 minutes (before eating) and 30 minutes after eating. The specificity remained at 100% at all time points, as determined by the cross-tabulation method.

DISCUSSION

Ghrelin is an orexigenic hormone that increases hunger and decreases food consumption. The literature has focused more on the entire ghrelin system than on the active form of the ghrelin hormone.[12-16] However, several studies have shown that gastric X/A-like cells secrete two forms of the ghrelin hormone: AG and DAG.[6] AG is the active form of the ghrelin hormone and is metabolised by GOAT to DAG, the inactive form of the ghrelin hormone. In theory, administering AG to animals and humans should increase food consumption, whereas administering DAG should decrease food consumption and suppress the functions of AG.[14,17-18] Therefore, AG is believed to play a role in the pathogenesis of obesity.

Obesity is the excessive accumulation of body fat often caused by an energy imbalance between calories consumed and calories expended. Obese individuals tend to

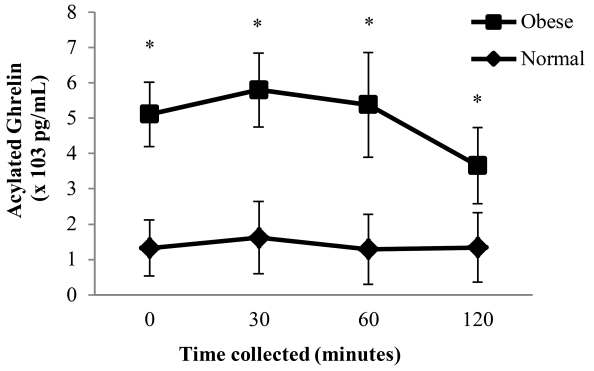


Figure 2. Acylated ghrelin (AG) concentrations before eating and 30, 60, and 120 minutes after eating. Data were analysed using an independent *t* test. AG concentrations were higher in obese individuals than in nonobese individuals ($p<0.01$). There was a significant difference (*) between the groups at each time point.

have heightened eating habits compared with individuals with normal BMIs.[19] This research revealed a significant difference between the AG concentrations in obese individuals and those in the control individuals. These significant differences are demonstrated at all time points, suggesting that obese individuals have higher AG. Although this study is the first to investigate the relationship between AG and appetite ratings among obese and normal weight men, several other studies have examined this relationship under different conditions. Lucio et al demonstrated that obese children without metabolic diseases had lower AG concentrations than normal weight children.[20] Zwirska et al investigated AG both pre- and postprandial in obese and normal weight women, revealing significantly higher concentration before eating in the obese participants.[15] This research exhibited similar results to those of Zwirska et al, with the exception of the sex of the participants.

According to the initial hypothesis, AG is believed to increase the risk of obesity. However, this contradicts the statement that ghrelin hormone decreases in obese individuals as compared with control individuals because of the body's compensation mechanism to diminish appetite.[5,7,12] The increase in AG and decrease in total ghrelin occur because the AG hormonal content circulating in the plasma is only 10% of the total existing ghrelin. The total ghrelin decrease can perhaps be attributed to the decrease of DAG in the plasma, which causes the energy balance to shift in a positive direction.[12] To understand this phenomenon, further research will be required, especially for DAG.

The graphic time curve of the AG concentration shows a steep increase in AG 30 minutes after eating for both the obese and control individuals. Spiegel et al monitored men of normal body weight and obtained similar results.[21] This relationship could be attributed to the mechanisms of the AG that structural resemblance to motilin which have increase appetite and gastroprokinetic activity.[18] The digestive process consists of four phases lasting between 30 and 40 minutes each, with the second and third phases involving an increase in gastric motility.[22]

This research also examined the differences in hunger and the desire to eat between obese and control individuals, correlating this subjective reporting to the objective assessment (AG) at different time points per group. Although many of the obese participants reported an excessive eating habit, no significant correlations were found.[19] Obesity is attributed to appetite increase; however, it is not necessarily influenced by hormonal factors alone. Genotype, fetal health, childhood experiences, social life (purchasing power), and environment are additional factors.[23] Obtaining quantitative data on the eating habits of obese and normal weight individuals in a research setting

Table 3. Independent *t* test of the area under the curve (AUC)

	Non obese individuals	Obese individuals	*p* value
AUC_{0-120} Acylated ghrelin (10^3) (pg.min/mL)	167±108	435±112	<0.01
AUC_{0-120} Hunger (10^3) (mm.min)	3.42±1.45	2.64±1.39	0.13
AUC_{0-120} Desire to eat (10^3) (mm.min)	4.11±1.87	3.50±2.35	0.51

Table 4. Relationship between appetite ratings and AG hormone content

Hormone	Appetite ratings	BMI	Time of measurement (min)	*p* value	Correlation coefficient
Acylated ghrelin	Hunger	Obese	0	0.84	-0.56
			30	0.66	0.12
			60	0.19	0.35
			120	0.36	0.24
		Normal weight	0	0.33	0.26
			30	0.83	-0.06
			60	0.41	-0.23
			120	0.87	0.05
	Desire to eat	Obese	0	0.19	0.34
			30	0.01	0.61
			60	0.36	0.24
			120	0.62	0.12
		Normal weight	0	0.82	0.06
			30	0.38	-0.24
			60	0.35	-0.25
			120	0.31	-0.27

Table 5. Cut-off, sensitivity, and specificity of AG content before and after eating at each time point

Time (min)	Cut-off (pg/mL)	AUC	Sensitivity (%)	Specificity (%)
0	2332	0.98	88	100
30	2710	0.96	88	100
60	2222	0.91	78	100
120	2222	0.77	52	100

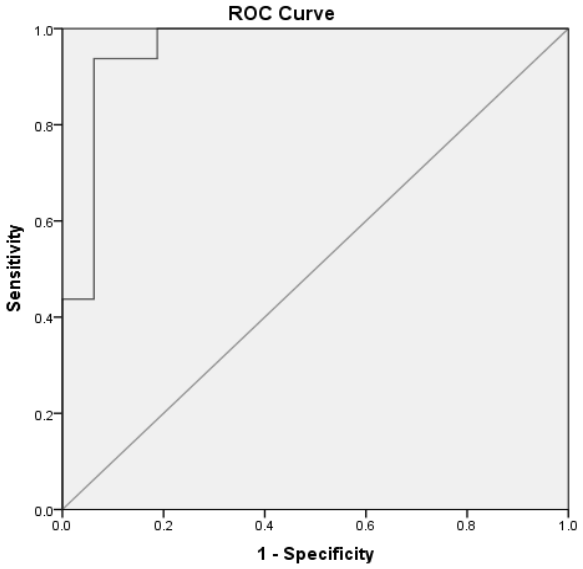
is difficult because participants consume food under strict protocols that may result in behaviours that differ from their natural eating habits.[24] Thus, it is reasonable for the relationship between the groups' eating habits not to conform with the hypothesis.

Acylated ghrelin plays a significant role in increasing food intake.[17] Recently, obesity therapy has been developed to inhibit GOAT. This inhibits the conversion of DAG into AG, resulting in decreased appetite and increased insulin secretion and sensitivity.[8] AG is also indirectly involved in the molecular mechanisms of type 2 diabetes mellitus. Thus, we sought to identify the AG cut-offs in our study. Rational therapy requires ascertainment of the AG cut-offs in obese and normal weight individuals.[25] In contrast to previous research assessing diagnostic tests for total ghrelin hormone, we observed AG in relation to hunger and the desire to eat.[13] Our diagnostic test revealed the baseline cut-off to be 0 minutes (before eating) to 30 minutes after eating, with a sensitivity and specificity of 88% and 100%, respectively. At time zero, the ghrelin is 2332 pg/mL, and at 30 minutes postprandial, the ghrelin is 2710 pg/mL.

A similar meal energy content used for in interventions did not influence the limited circadian rhythm of AG and showed a similar appetite response in both group. However, the concentration of AG was still higher in the obese than in the normal weight individuals.

In conclusion, we show that plasma AG concentration is higher before and after eating at all time points, regardless of food type, in obese individuals, as compared with normal weight individuals. The observed increase of AG in obese individuals indicates that the actual decrease of total ghrelin in obese individuals could be an attempt to reduce the progress of obesity. However, an increased AG, which is only 10% of total ghrelin, masks the body's attempt to compensate for obesity. Thus, a decrease in total ghrelin may not limit the progress of obesity (Figure 4).

A. Before eating



B. 30 minutes after eating

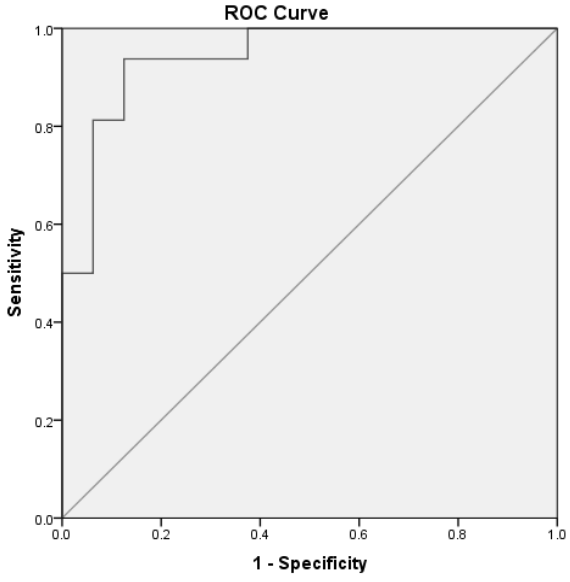


Figure 3. Receiver operating characteristic curve of AG: (A) before eating and (B) 30 minutes after eating.

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AUTHOR DISCLOSURES

The authors declare that they have no conflicts of interest.

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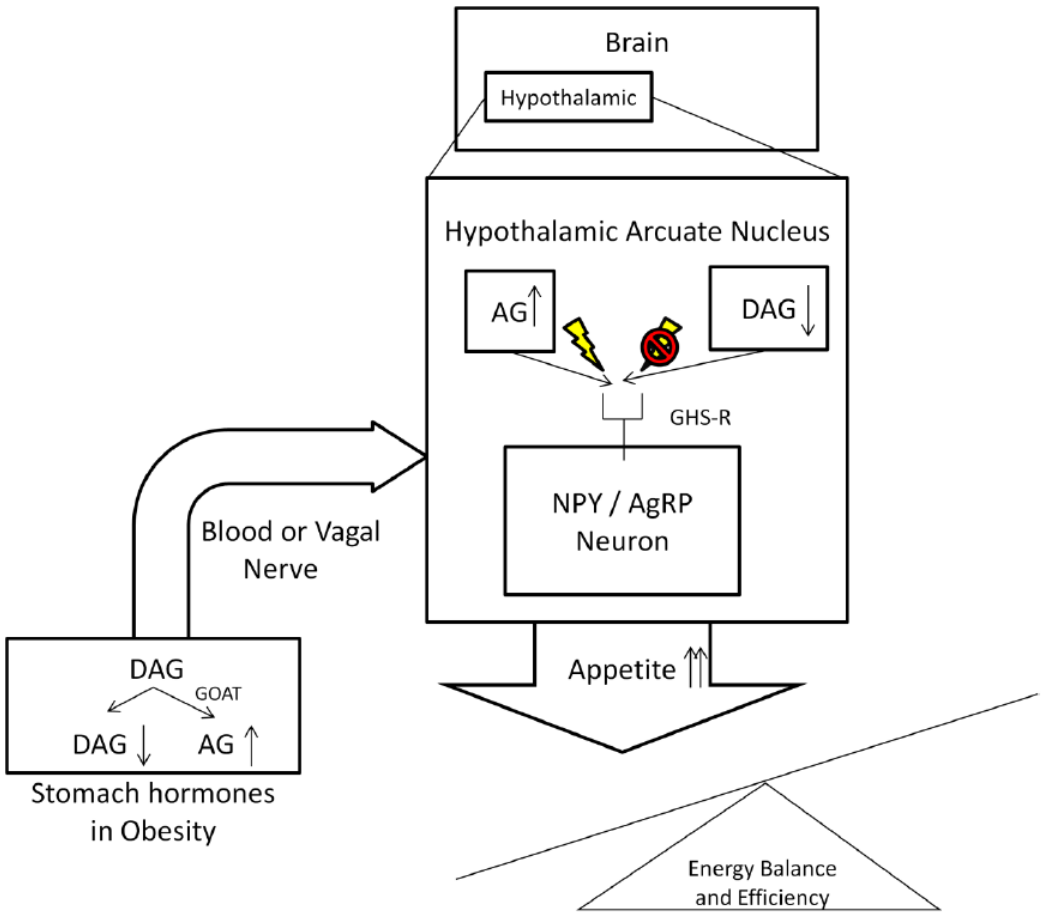

Figure 4. The appetite hormone ghrelin and its acylation in the stomach for energy balance. AG: acylated ghrelin; AgRP: agouti-related protein ; DAG: deacylated ghrelin; GHS-R: Growth hormone secretagogue receptor; NPY: neuropeptide Y.

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