ORIGINAL ARTICLE

Ghrelin – growth hormone releasing and orexigenic hormone in men declines with age, insulin and with decrease in testosterone concentration

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Abstract

OBJECTIVES. Ghrelin, a natural ligand of the growth-hormone secretagouges receptor is the peptide known because of its growth hormone (GH)-releasing as well as orexigenic actions.
THE AIM of the study was to examine the relationship between ghrelin levels and age, weight, glucose metabolism markers, GH, IGF-1 and androgens in men.
PATIENTS AND METHODS. The study included 19 healthy men aged 16–73 yrs, mean 46,8 ±18,5 (mean ± SD). The height, weight, and body mass index (BMI) of the study subjects were assessed and serum concentration of fasting ghrelin, leptin, GH, IGF-1, glucose, insulin, estradiol, testosterone and DHA-S were measured.
RESULTS. The negative correlation between age and serum ghrelin levels (r=–0,52; p<0,04) in men was found. In men under 30 years old serum ghrelin levels were significantly higher compared to men over 60 years old (p<0,02). Serum ghrelin levels in men were significantly lower compared to healthy women aged 43,5 ± 12 years old (p<0,008). Serum insulin levels correlated negatively with ghrelin levels (r= –0,87; p<0,0006) and serum testosterone concentrations correlated with ghrelin levels (r= 0,69; p<0,00006).
CONCLUSION. Our findings demonstrate that the serum ghrelin levels in men decline with age, is lower than in women and correlate negatively with fasting insulin levels. We demonstrated, for the first time, that serum testosterone levels in men correlate with total ghrelin levels.

INTRODUCTION

Ghrelin was discovered in 1999 as a natural ligand of growth hormone secretagouges receptor type 1a ("G-protein coupled", GHS-R), which activation is able to produce a strong stimulating effect on GH secretion in rodents and in humans (Bowers. 2001). Ghrelin is 28-amino acid peptide, in which the serine-3 residue is *n*-octanoylated (Kojima *et al.* 1999). It is predominantly produced by the stomach, but also is secreted in small amounts by the intestines, kidneys, pituitary, hypothalamus and placenta (Date *et al.* 2000). The stimulatory effect of ghrelin on GH secretion is more potent then that of GHRH, although data from studies in rodents and in humans indicate that ghrelin is involved in GH-regulating system along with GHRH (Hataya *et al.* 2001). In cases of GH hypersecretion (acromegaly)

Abbreviations

AGRP	– agouti-related protein
BMI	– body mass index
DHA-S	– dehydroepiandrosterone-sulfate
IGF-1	– insulin-like growth factor 1
GH	– growth hormone
GHRH	– growth hormone releasing hormone
GHS-R	– growth hormone secretagouge receptor
GHS-R1	– growth hormone secretagouge receptor type 1
hCG	– human CG
HSD	– hydroxy steroid dehydrogenase
MC34	– melanocortin 3 receptor
MC4R	– melanocortin 4 receptor

serum ghrelin levels are decreased (Kozakowski *et al.* 2005, Waśko *et al.* 2006), although some authors haven't found differences between serum fasting and during oral glucose tolerance test ghrelin levels in acromegalic patients and healthy controls (Isidro *et al.* 2007). It has been demonstrated, that ghrelin mRNA and peptide are expressed not only in normal but also in adenomatous human pituitary tissue (Korbonits *et al.* 2001, Kim *et al.* 2001). Authors that have not detected ghrelin mRNA in somatotroph adenoma tissue obtained at the time of transsphenoidal surgery have postulated, that absence of the peptide is mainly due to the preoperatively treatment with somatostatin analogues (Waśko *et al.* 2006).

In addition to GH-releasing properties ghrelin exerts orexigenic effects. Since GHS receptors have been found on arcuate hypothalamic neurons involved in the control of feeding behavior it has been proposed that ghrelin participates in the regulation of energy balance by stimulation of food intake. In addition, it has been demonstrated that ghrelin is able to inhibit lipids oxidation and then induces adiposity. It is called a "hunger hormone" and correlates negatively with "satiety hormones" such as insulin and leptin (Broglio *et al.* 2001, Toshinai *et al.* 2001). Both central and peripheral administration of ghrelin stimulates food intake (Shintani *et al.* 2001).

In addition to endocrine and metabolic actions ghrelin has been found to have inhibitory effects on inflammatory processes. This finding suggests possible involvement of the peptide in the pathogenesis of inflammatory disorders, e. g. the inflammatory demyelinating disease of the central nervous system, multiple sclerosis (Berilgen *et al.* 2005).

It has also been demonstrated, that injection of ghrelin increases markedly activity of lysosomal enzymes in blood serum in rabbits (Witek *et al.* 2005). It is well known, that lysosomal system is designed not only for intracellular digestion, but it also plays an important role in the adaptation processes.

Recent studies claim that ghrelin influences the androgen levels in animals and in humans. The ability of ghrelin to modulate stimulated testosterone secretion *in vitro* has been presented in rats (Tena-Sempere *et al.* 2002). The examination of the expression of ghrelin in testicular tissues in men have shown, that ghrelin expression by Leydig cells has been inversely correlated with the serum testosterone concentration, although has not been directly related to spermatogenesis (Ishikawa *et al.* 2007). In women with polycystic ovary syndrome (PCOS) ghrelin levels have been negative correlated with testosterone (Bik *et al.* 2007). However, other authors didn't find the correlation between ghrelin levels and testosterone, androstendione, dehydroepiandrosterone-sulfate and 17-OH-progesterone levels in women with PCOS before and after 6-months therapy with cyproterone acetate (Kos-Kudła *et al.* 2006)

It is assumed that the biological activity of ghrelin is dependent on its acylation, although recent data show that non-acylated form is not biologically inactive and shares some metabolic and cardiovascular actions of active hormone. It has been found that relation of octanoylated to deoctanoylated form of ghrelin in stomach is roughly 1:3, and that acylated ghrelin circulates at 2,5-fold lower concentrations than unacylated form (Kojima *et al.* 2002).

In humans circulating ghrelin levels vary acutely and chronically with nutritional status; levels are elevating in fasting and fall after food intake (Shiiya *et al.* 2002). However, little is known by now about depending on age, gender and anthropometric as well as metabolic markers of ghrelin in humans.

The aim of the study was to examine the relationship between ghrelin levels and age, body weight, glucose metabolism markers, growth hormone, IGF-1 and androgens in men.

MATERIALS AND METHODS

The study included 19 healthy men aged 16–73 yrs, mean 46,8 ± 18,5 (mean ± SD) with a stable weight, on no medications, no smoking, non-dieting, without any eating disorder. A screening session included a full physical examination and routine laboratory tests. Subjects were studied after an overnight fast. They underwent assessment of body height and weight, and then body mass index (BMI) was calculated. Blood was collected at 0900 h for total ghrelin, GH, insulin-like growth factor I, glucose, insulin, estradiol, testosterone and dehydroepiandrosterone-sulfate measurements through an iv catheter placed in the forearm.

The local ethical committee approved the study and informed consent was obtained from all the participants.

Assays

Total ghrelin was measured with a commercial RIA (Phoenics Phamaceuticals, Inc, Belmont, CA) This RIA uses J^{125}-labeled ghrelin as a tracer and polyclonal antibody raised in rabbits against full-length, octanoylated human ghrelin that recognizes both the octanoyl and des-octanoyl forms of the hormone. The sensitivity for this assay was 100 pg/ml. The intraassay coefficient of variation was 4%. The interassay coefficient of variation was 10%. Leptin was measured by RIA (Phoenics Phamaceuticals, Inc, Belmont, CA) using polyclonal antibody raised in rabbits against leptin. The sensitivity for this assay was 0,5 ng/ml. Growth hormone was measured

Table 1. Anthropometric characteristics of study subjects.

No		n	X ± SD	Range
1	Age (yrs)	19	48,8 ± 18,5	16, 0 – 73, 0
2	Height (m)	19	1,75 ± 0,08	1,61 – 1,90
3	Weight (kg)	19	81,4 ± 15,1	54,2 – 116,0
4	BMI (kg/m2)	19	26,6 ± 3,9	21,1 – 37,5

BMI, body mass index

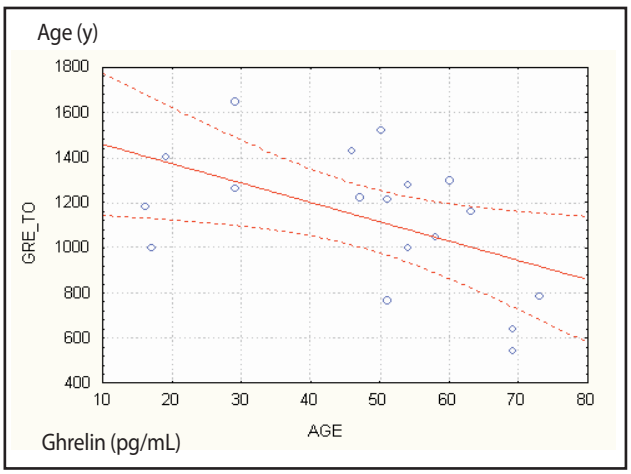

Fig. 1. Correlation between age and ghrelin levels, r= -0,52; p<0,04

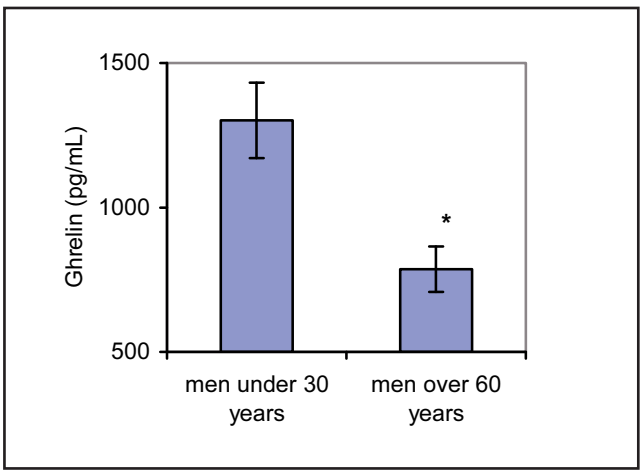

Fig. 2. Ghrelin levels in men under 30 years and over 60 years old (*p<0,02).

by RIA (Polatom, Otwock-Świerk, Poland); sensitivity of this method was 10,6 μg/ml. Insulin growth factor-1 was measured by RIA (Biosource Europe S. A, Belgium); sensitivity was 0,25 ±0,1 μg/ml. Insulin was measured by RIA (INSULIN-CT, CIS bio international, France); sensitivity was 2,0 mIU/ml. Glucose was measured by enzymatic method (Cormay). Total cholesterol and triglycerides were measured by enzymatic method (Technikon, RA-390, Boeringer). Estradiol was measured with IMMULITE 2000 analyzer; sensitivity was 15 pg/ml. Testosterone was measured by RIA method (Polatom, Otwock-Świerk, Poland); sensitivity of this method was beyond 0,1 nmol/L, the intra- and interassay coefficients of the variations were 6,6% and 4,8%, respectively. Dehyrdroepiandrosterone-sulfate was measured by RIA method (Spectria, Orion Diagnostica, Finland); sensitivity of this method was 0,03 μmol/L, the intra- and interassay coefficients of the variations were 6,5% and 8,1%, respectively. Hypogonadism was diagnosed if serum testosterone level was lower than 3,5 ng/mL.

Body mass index was calculated as a body weight (kg)/height (m^2). Subjects with BMI lower than 25 kg/m^2 were considered as having a normal weight whereas subjects with BMI between 25 and 30 kg/m^2 were considered as overweighed, with BMI between 30 and 40 kg/m^2 as obese, and with BMI above 40 kg/m^2 as morbidly obese.

Statistical analysis

All data are presented as mean ± SD. We verified the normality of the distribution of variables with a Kolmogorov-Smirnov and Lilieforse tests. To examine relationship between data Pearson correlation or Spearman rank analyses were used. Comparisons between groups with normal distribution of data were performed by unpaired Student's t-test, in other cases comparisons were performed by Kolmogorov-Smirnov test for two samples. P level <0,05 was considered statistically significant.

RESULTS

Table 1 shows results of anthropometric measures and body mass index in men participated in the study. This cohort represented a broad range of age and body weight. Eleven subjects had normal weight, six were overweighed and two were considered as obese.

GH axis hormones, leptin, carbohydrate metabolism markers and serum lipids in study subjects are presented in Table 2. Three men were hypecholestrolemic and two had triglicerydes levels above the normal range. Serum fasting IGF-1 levels correlated negatively with age (r= –0,88, p=0,003).

Circulating estradiol and androgens levels are shown in Table 3. Three men fulfilled criteria of hypogonadism, the reminder men participated in the study were eugonadal. There was negative correlation between age and serum testosterone levels (r= –0,51, p<0,04).

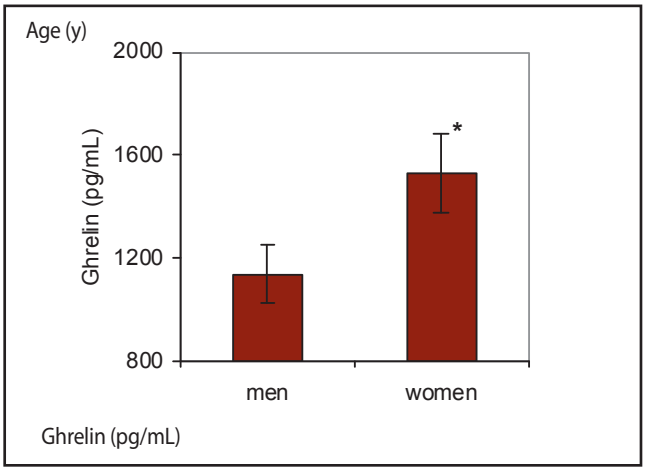

Fig. 3. Ghrelin levels in study men and in group of healthy women aged 43,5 ± 12 years old (*p<0,008).

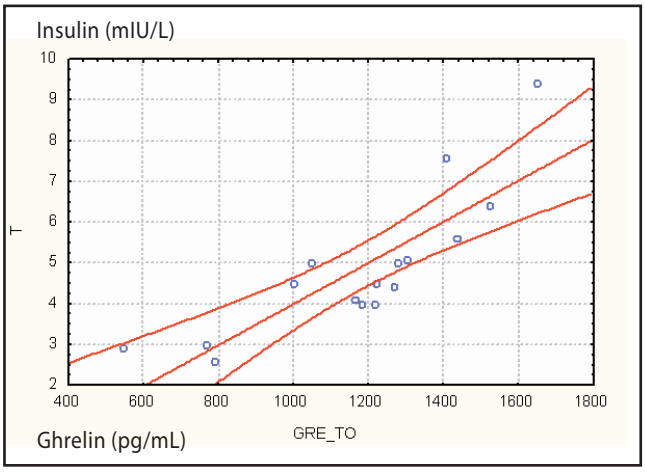

Fig. 4. Correlation between serum fasting insulin and ghrelin levels, r= -0,70; p<0,02.

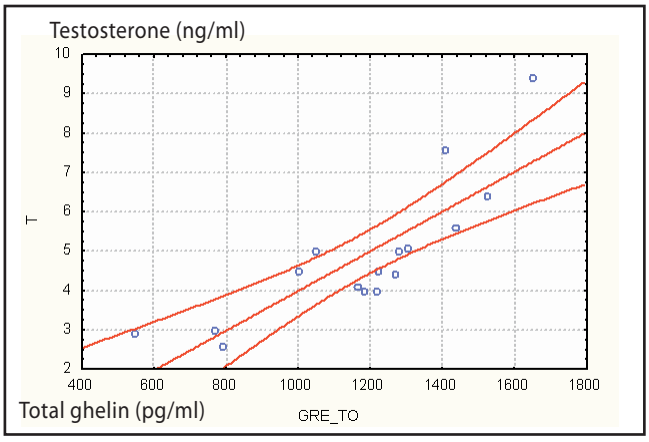

Fig. 5. Correlation between serum fasting total testosterone and ghrelin levels, r= 0,69; p=0,00006.

Table 2. GH, IGF-1, leptin, carbohydrate metabolism markers and serum lipids levels in study subjects

No		n	x ± SD	Range
1	Glucose (mmol/l)	13	4,94 ± 0,4	4,39 – 5,55
2	Cholesterol (mmol/l)	10	4,90 ± 1,2	2,7 – 6,7
3	Triglicerydes (mmol/L)	8	1,67 ± 0,85	0,88 – 2,85
4	Leptin (µg/L)	17	6,8 ± 6,3	1,3 – 26,0
4	Insulin (mIU/L)	12	19,4 ± 3,3	10 – 21
5	GH (µg/dL)	12	0,50 ± 0,3	0,3 – 1,1
6	IGF-1 (µg/L)	12	339,0 ± 73,0	270 – 450

GH, growth hormone; IGF-1, insulin-like rowth factor 1

Table 3. Estradiol, testosterone and DHA-S serum levels

No		n	x ± SD	Range
1	Estradiol (pg/mL)	7	24,0 ± 7,6	15,0 – 33,3
2	Testosterone (ng/mL)	17	4,88 ± 1,7	2,6 – 9,4
3	DHA-S (ng/mL)	14	2084 ± 1509	141 – 4385

DHA-S, dehydroepiandrosterone-sulflate

Fasting serum ghrelin levels in men ranged from 549 to 1647 pg/mL; the mean value was 1136,2 ± 301 pg/mL. There was negative correlation between age and ghrelin levels in men (r=–0,52; p<0,04; Fig. 1)

In men under 30 years old serum ghrelin levels were significantly higher compared to men over 60 years old: 1301,8 ± 241,6 pg/mL to 785,7 ± 271,3 pg/mL, respectively, p<0,02. (Fig. 2)

Fasting serum ghrelin levels in study men were significantly lower compared to group of ten healthy women aged from 28 to 65 years old (mean 43,5 ± 12): 1136,2 ± 301 pg/mL to 1528,2 ± 384 pg/mL, respectively, p<0,008 (Fig. 3)

In study subjects we found tendency towards negative correlation between body mass index and total ghrelin levels (r= –0,38; p= 0,12). The correlation between BMI and leptin levels was also found (r= 0,58; p<0,02).

There was also strong negative correlation between serum fasting insulin and ghrelin levels (r= –0,87; p<0,0006; Fig. 4).

We found strong correlation between serum fasting total testosterone and ghrelin levels (r= 0,69; p=0,00006, Fig. 5).

DISCUSSION

The aim of our study was to determine if there were any relationships between fasting ghrelin levels and age, body weight, growth hormone axis, estradiol, androgens and glucose metabolism markers in men.

We found that in men serum ghrelin levels decreases with age, and we demonstrated tendency towards a negative correlation between serum ghrelin levels and body mass index. In our subjects ghrelin levels correlated negatively with serum insulin levels. We also found significant relationship between serum testosterone and ghrelin levels.

It is well known that ghrelin levels in humans decline with age (Rigamonti *et al.* 2002). Supporting this concept our results demonstrate that ghrelin levels indeed decline with age in men. It is interesting to speculate whether decline in growth hormone secretion during aging ("somatopause") may be at least in part caused by decreasing ghrelin production. However, we did not demonstrate any significant relationships between ghrelin and GH or IGF-1 serum levels. These observations should be confirmed in the next studies.

Although some studies in rodents and humans have shown that serum ghrelin levels are gender-dependent (Greenman *et al.* 2004, Liu *et al.* 2002) we for the first time demonstrated that serum ghrelin levels in healthy men were lower than in women. This finding probably at least in part explains reasons of higher growth hormone serum levels in women than in men. Hitherto we knew, mostly from animals studies, that estrogens likely stimulate GH release via effects on hypothalamic somatostatin and GHRH secretion. Reduced negative feedback by IGF-1 was also taken into consideration.

We also demonstrated that there was trend towards negative correlation between body mass index and ghrelin levels and direct correlation with leptin levels. In the present series the correlation between BMI and ghrelin did not reach significance. It is however possible, that variability among our patients in other determinants of circulating ghrelin levels could have obscured such relationship. Data on correlation with leptin are in accordance with previous studies investigating this question and support concept that both hormones participate in regulation of energy balance. The concentration of circulating ghrelin, principally derived from the stomach is influenced by changes in nutritional state. Fasting increases plasma ghrelin, which crosses blood-brain barrier and activates hypothalamic neuropeptide Y/agouti gene-related protein (AGRP) neurons of ventromedial and arcuate nucleus (Coiro *et al.* 2006) and increases food intake and, in consequence, body weight. Increase in fat mass causes in turn rise in leptin production. Leptin, a product of ob gene carries the information about adipose tissue mass to hypothalamic neurons involved in the control of feeding behavior. As the consequence of increase in fat mass neuropeptide Y and AGRP expression in suppressed and proopiomelanocortin expression is increased with subsequent activation of MC3R and MC4R receptors what contributes to feeling of satiety and decrease in food intake (Horvath *et al.* 2001, Shintani *et al.* 2001). We also demonstrated in our study, that serum leptin levels rose alongside with increase in body weight and body mass index. Our data are in agreement with results of the other authors (Gomez *et al.* 2003, Kondo *et al.* 2002). On the other hand it is known that regular exercising that reduces body fat rate also reduces serum leptin levels (Unal *et al.* 2005)

The strong negative correlation between insulin and ghrelin levels has already been shown and was confirmed by the present study. The decrease in plasma ghrelin levels was paralleled by an increased of plasma insulin. The changes in serum insulin levels before and after meal that were virtual mirror images of fluctuations in ghrelin levels have also been shown (Cummings DE *et al.* 2001, Cummings DE *et al.* 2002). Additionally some data has shown that insulin is a physiological modulator of serum ghrelin concentration and that insulinaemia possibly mediates the effects of nutritional status on its concentration (Saad *et al.* 2002).

It was interesting although not very surprising to find significant correlation between serum levels of ghrelin and testosterone. It is known that testosterone production is at least partially ghrelin-dependent. Evidence for the expression of ghrelin and its receptor in rat Leydig cells, i.e. steroidogenic cell-type was provided in 2002. As it was demonstrated in experiment *in vitro* ghrelin may inhibit hCG- and cAMP-induced testosterone secretion in dose-dependent manner. Decreased testosterone production in response to ghrelin was associated with significant decrease in hCG-stimulated expression levels

of the mRNAs of key enzymes of steroid biosynthesis: 3β-HSD and 17β-HSD type III, although causative relationship between these phenomena were not proven. Authors speculate that ghrelin may be an important factor in the endocrine network connecting control of reproductive system and the energy balance (Tena-Sempere *et al.* 2002). In the other study authors (Barreiro *et al.* 2002) demonstrated that ghrelin selectively located in Leydig cells at advances stages of maturation is under hormonal regulation of LH. It is not known whether ghrelin in testis is also a regulator of Sertoli cells function, but lack of effect of administration of FSH on ghrelin mRNA doesn't suggests that Sertoli cell factors may be involved in the regulation of testicular ghrelin expresion. It seems, that ghrelin may operate in testis as a local regulator of the steroidogenic actions of LH, participating in the autolimitation of the testosterone response to gonadotropic stimulation. It can't be excluded, that ghrelin is a mediator of LH actions other than steroidogenic effects. Also in human testis evidence for the presence of both components (ligand and receptor) of the ghrelin signaling has been demonstrated using molecular and immunohistochemical methods. Ghrelin was detected in Leydig and in Sertoli cells. In addition to the ligand, GHS-R immunoactivity was observed in human testis in wide pattern in germ cells, mainly in pachytene spematocytes as well as in Leydig and Sertoli cells (Gaytan *et al.* 2004). The simultaneous expression of both components of this signaling suggests that in men ghrelin may play a role in the testosterone production and in the paracrine control of seminiferous tubule function. Additionally, it can't be excluded, that circulating ghrelin may operate upon specific testicular cell targets. In the study from 2003 authors demonstrated that serum ghrelin levels were significantly lower in hypogonadal men and increased after hormonal replacement therapy with testosterone. A significant positive correlation had also been found between ghrelin and total and free testosterone (Pagotto *et al.* 2003). Our data extend those observations and demonstrate similar relationship in a group of healthy men in wide range of age. In our study we evaluated serum ghrelin and testosterone levels. It is not known to what degree circulated ghrelin participates in local regulation of testosterone production.

We dind't find significant correlation between serum ghrelin and gonadotropins levels. Lack of this correlation may indicate that locally produced but not circulated ghrelin is mainly involved in physiological regulation of steroidogenesis in human testis. Further studies are necessary to confirm and elucidate the relationship between testosterone and locally produced as well as circulated ghrelin.

In summary, the present study demonstrates that the serum ghrelin levels in men decline with age, is lower than in women and correlate negatively with fasting insulin levels. We also demonstrated, for the first time, that serum testosterone levels in men correlate with ghrelin levels.

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