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ORIGINAL ARTICLE

Alcohol dependence is associated with reduced plasma and fundic ghrelin levels

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ABSTRACT

Background Conflicting data concerning the involvement of ghrelin in the pathophysiology of alcohol dependence have been reported. The aim of this study is to investigate how chronic alcohol ingestion influences plasma ghrelin levels and whether potential changes observed in plasma relate to modifications in ghrelin production in the stomach where this peptide is primarily synthesized.

Materials and methods Fifty-one consecutive alcoholics admitted for alcohol withdrawal were prospectively enrolled and compared to a control group of 32 healthy volunteers matched for age, sex, height and weight. All subjects underwent fasting plasma ghrelin determination. Twenty-seven randomly selected alcoholics and 17 controls underwent gastroscopy for fundic and duodenal biopsies. Tissues were fixed for histology or frozen in liquid nitrogen for ghrelin protein and mRNA determinations by a radioimmunoassay and quantitative polymerase chain reaction, respectively. Alcohol consumption was normalized to body weight (BW) or body mass index (BMI) given the influence of BW and volume distribution on alcohol levels.

Results Plasma and fundic ghrelin protein levels were significantly decreased in alcoholics. Fundic but not plasma ghrelin protein levels inversely correlated with alcohol consumption normalized to BW or BMI. Ghrelin mRNA levels in fundic biopsies were similar in alcoholics and controls. No significant differences in duodenal ghrelin protein and mRNA levels were found between both groups.

Conclusions Alcoholism was associated with decreased plasma ghrelin levels partly due to reduced ghrelin production in the stomach. Alcohol affected ghrelin production on the post-transcriptional level in the fundus, whereas duodenal ghrelin secretion did not respond in a similar manner to alcohol intake.

Keywords Alcoholism, blood, duodenum, ghrelin, stomach.

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Introduction

Ghrelin is a peptide hormone which has several biological activities including stimulation of growth hormone secretion and regulation of appetite and the energy balance. This peptide hormone is mainly synthesized by the endocrine cells of the gastric fundic mucosa and the intestine [1,2]. However, additional sites of secretion have been described like the hypothalamus, pituitary, liver, pancreas, kidney, placenta and mononuclear cells of the immune system [3,4]. The gene that encodes ghrelin is located on chromosome three in man [5]. Ghrelin is initially synthesized as a pre-pro-hormone which is subsequently proteolytically processed to produce a 28-amino-acid peptide. A modification on the peptide hormone during synthesis occurs in the form of an octanoyl group on serine three, which is essential for some of its biological activities. Ghrelin is the endogenous ligand for the growth hormone secretagogue (GHS) receptor which is able to stimulate food seeking behaviour [6,7]. The existence of a gastrointestinal hypothalamic–pituitary–adrenocortical axis, responsive to nutritional and caloric intakes [4], that would play a role in GH secretion, body growth and appetite has been proposed.

Several studies have suggested that ghrelin might play a significant role in the pathophysiology of alcoholism together with another peripheral peptide leptin. The effect of the adipocyte secreted anorexigenic peptide leptin is antagonized by the action of ghrelin [8,9]. The actions of ghrelin and leptin seem to be mediated centrally by the neuropeptide Y and the Agouti-related protein system both related to the central regulation of energy homeostasis [10,11]. Food seeking behaviour and alcohol seeking behaviour are related to common underlying neural circuits [3,12,13].

It has been shown that alcohol intake has an acute inhibitory effect on ghrelin secretion in normal man [14]. In addition, fasting plasma ghrelin levels increase during the period of alcohol

abstinence. However, there is an ongoing debate as to whether chronic active drinking decreases or increases plasma ghrelin levels [7,8]. Especially, it remains to be determined whether active drinkers have reduced ghrelin levels compared to normal non-drinking subjects and if such a change is confirmed, to understand where the modification of ghrelin secretion occurs in chronic alcoholism.

To answer these questions, the aims of the present study were to investigate whether chronic alcohol ingestion influences plasma ghrelin levels compared to healthy subjects (social drinkers) and to define whether the potential modifications relate to changes of ghrelin production in the stomach.

Materials and methods

Study population and study design

Fifty-seven consecutive patients responding to the DSM IV criteria for alcohol dependence [15], who were admitted to the hepatology unit for alcohol withdrawal were prospectively included. Diazepam was administered orally on an individual basis within 24 h of admission according to the occurrence of withdrawal symptoms and all alcoholics were supplemented with oral vitamin B1, B6 and B12. No anti-craving medication was used during hospitalization. Patients with cirrhosis, known diabetes, abnormal fasting glucose levels, abnormal fundic or duodenal histology, or who had stopped drinking for more than 24 h prior to admission were excluded from the study. Accordingly, six patients had to be excluded: four patients for discovery of cirrhosis, one patient for de novo diabetes, and one for severe gastritis on histology leaving a final study population of 51 alcoholic subjects. A complete dietetic investigation and assessment of alcohol consumption were performed by a specialized dietician using a standardized semi-structured questionnaire that has been adapted to alcoholic patients and internally validated in our institution. In this routinely used questionnaire, patients are asked to recall what they have eaten and drunk (including alcoholic beverages) during each meal and between meals of each day of the week preceding their admission to the unit. All patients underwent a detailed physical examination. On the second day of hospitalization, a blood sample for fasting plasma ghrelin levels and routine laboratory testing was taken in all patients. On the same day 27 randomly selected subjects among the 51 alcoholic patients also underwent an upper gastrointestinal (GI) endoscopy for standard fundic and duodenal biopsies for determination of tissue ghrelin levels and routine histology.

Alcoholic patients were compared to a control group of 38 healthy non-alcoholic volunteers who were matched for age, height and weight. A blood sample for fasting plasma ghrelin was taken in all healthy, non-alcoholic volunteers. Although all 38 volunteers had a dietetic history and blood samples taken according to the protocol, reliable plasma ghrelin levels could not be obtained in six volunteers even on repeat measurements due to interference with a high radioactive background on the ghrelin RIA assay. It was therefore decided not to consider them for further analysis leaving a final study population of 32 volunteers. Seventeen of them also underwent upper GI endoscopy in the outpatient endoscopy unit where standard fundic and duodenal biopsies for ghrelin measurements and histology were taken during the procedure. The principal indication for upper GI endoscopy was gastro-oesophageal reflux symptoms in alcoholics and healthy volunteers. None of the subjects finally included in the study showed any significant histological changes or damage on standard microscopic examination of the fundic and duodenal biopsies.

For all participants age, sex, weight and height were recorded and body mass index (BMI) was calculated. Written informed consent was obtained from all patients and volunteers. The study protocol was approved by the Ethics Committee of the Université Catholique de Louvain, in Brussels, Belgium.

Analytic methods

Specimen collection and storage

All samples were collected in the morning from fasting patients who, given the influence of smoking on ghrelin levels, were smoke free for at least 8 h. [16]. In order to assure stability of the active form of ghrelin protein in plasma, blood samples were kept on ice before being rapidly centrifuged at 3000 rounds per minute for 7 min at 4 °C. Thereafter, plasma was treated by acidification with 50 μL of 1 N HCl followed by the addition of a commercially available protease inhibitor cocktail (Roche Diagnotics, Vilvoorde, Belgium) and 10 μL of phenylmethylsulfonyl fluoride (PMSF) per 1 ml of plasma. Samples were stored at –20 °C until analysis. Tissues specimens were immediately frozen in liquid nitrogen for ghrelin protein and mRNA determinations. Additional samples were fixed in Bouin solution for routine histological examination by an experienced pathologist.

Ghrelin and mRNA extraction

Ghrelin extraction from tissues was performed according to the method described by Hofbauer *et al*. [17]. Total RNA was prepared from frozen tissue using TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) following the instructions of the manufacturer.

Determination of ghrelin protein and mRNA

Ghrelin mRNA levels were measured by quantitative polymerase chain reaction (PCR). Quantitative PCR analysis was performed with the GeneAmp® 5700 Sequence Detection System and

software (Applied Biosystems, Den Ijssel, Netherlands) using Cybergreen fluorogenic probes. Ribosomal protein L19 (Rpl19) RNA was chosen as an internal standard. All primers were designed using the Primer Express™ design software (Applied Biosystems). The following primers were used: ghrelin 5′-3′ GGCAGGCTCCAGCTTCCT, 3′-5′-GGTGGCTTCTTCGACTCCTTT; Rpl19 5′-3′ CAAGCGGATTCTCATGGAACA, 3′-5′-TGGTCAGCCAGGAGCTTCTT. PCR reactions were performed according to the standardized thermal profile of the system previously set by the manufacturer. All tissue samples were run in duplicate at the same time in a single 96-well reaction plate (MicroAmp® Optical, Applied Biosystems) using appropriate primers and probes. Quantification was obtained according to the ΔCT method as specified by the manufacturer. The final result of each sample was normalized to its respective Rpl19 value.

Plasma and tissue ghrelin protein levels were determined by a radioimmunoassay (RIA) kit (Linco, St. Charles, MO, USA) following the instructions of the manufacturer. The RIA kit uses an antibody which is specific for the biologically active form of ghrelin with the octanoyl group on serine three.

Statistical methods

After having examined whether the results are normally distributed (Kolmogorov–Smirnov test), a Student's *t*-test was performed to assess significance. A *P*-value < 0·05 was considered statistically significant. Data were expressed as mean ± standard error of the mean (SEM). Median and range is also given for demographic data as well as nutritional and alcohol intake. Correlations were determined using the Spearman correlation test.

Results

Demographic data, nutritional intake and alcohol consumption

The principal demographic data are summarized in Table 1. Overall both groups were well balanced in terms of age, weight, height and BMI. There was a slight difference in male-female sex ratio with males being over-represented in the alcoholic group compared to the healthy volunteers. The total amount of kCal day^{-1} as well as the relative proportion of proteins, lipids and carbohydrates that constitute the total nutritional intake is summarized in Table 1 for both groups. All alcoholic patients included in the study had normal fasting glucose levels (normal range 80–100 mg dL^{-1}). Mean daily alcohol intake was 1026·69 ± 763·39 kCal day^{-1} (144·93 ± 87·29 g alcohol day^{-1}). Physiological consequences of alcohol may likely depend on blood alcohol levels that are influenced by the body weight and the volume distribution of an individual patient. To take into account this aspect during analysis of the data, we normalized the alcohol intake to body weight and BMI in alcoholic patients (Table 1). Healthy volunteers were social drinkers consuming less than 30 g ethanol per day. Their mean daily alcohol intake was 103·72 ± 96·81 kCal day^{-1} (14·82 ± 13·83 g alcohol day^{-1}) (Table 1).

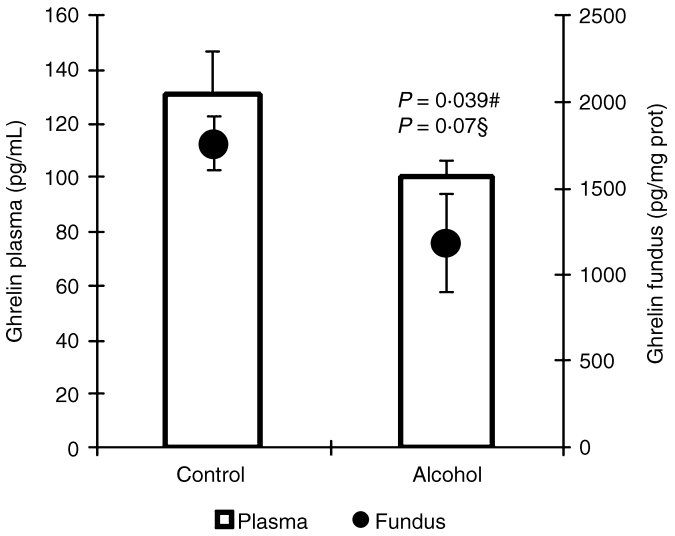

Figure 1 Plasma and fundic ghrelin protein levels in healthy controls and in alcoholic patients. Biologically active plasma ghrelin levels (columns) were significantly decreased in alcoholic patients ($n = 51$) compared with controls ($n = 32$). A very similar trend of reduced ghrelin protein levels (dots) was found in fundic biopsies of alcoholic patients ($n = 27$ vs. $n = 14$ in controls). Indicated are means ± SEM. *P*-values compare alcoholics and controls # in plasma and § in the fundus.

Ghrelin protein levels

Plasma ghrelin levels were significantly decreased in alcoholic patients compared with healthy controls (99·9 ± 6·4 vs. 131 ± 15·8 pg mL^{-1}; $P = 0·039$; $n = 51$ vs. $n = 32$ subjects, respectively) (Fig. 1). In parallel, a clear trend towards decreased ghrelin protein levels was observed in fundic biopsies in alcohol dependent patients compared with healthy controls (1183 ± 158 vs. 1755 ± 289 pg mg^{-1} protein; $P = 0·07$; $n = 27$ vs. $n = 14$ subjects, respectively). However, this difference did not reach statistical significance (Fig. 1).

Overall ghrelin expression was low in duodenal biopsies compared with fundic biopsies in both groups. Ghrelin protein levels tended to be higher in duodenal biopsies from alcoholic patients compared with controls (Table 2) suggesting that ghrelin secretion in the duodenum responds differently to high alcohol intake in comparison with ghrelin secretion in fundus.

Table 1 Principal demographic data, nutritional and alcohol intake in the study groups

Demographic data	Control group ($n = 32$) Median (range) Mean ± SEM	Alcoholic group ($n = 51$) Median (range) Mean ± SEM	*P*-value
Age (years)	44 (21–73)	46 (28–71)	P = NS
	43·9 ± 2·4	47·2 ± 1·5	
Sex ratio M/F	15/17	29/22	P = NS
Weight (kg)	69·5 (47–99)	71·5 (47–103)	P = NS
	71·1 ± 2·4	72 ± 2·1	
Height (cm)	171 (156–199)	171 (150–189)	P = NS
	173 ± 2	171 ± 1	
BMI (kg m^{-2})	24 (18–30·2)	24·1 (16·1–36·2)	P = NS
	23·8 ± 0·8	24·5 ± 0·6	
kCal intake			
Total kCal day^{-1}	2378 (1484–4392)	2464 (1073–6193)	P = NS
	2407 ± 203	2746 ± 176	
Protein (% total kCal $day)^{-1}$	15·9 (11·3–20·4)	10·5 (4–23·2)	$P < 0·001$
	16·6 ± 0·6	11·1 ± 0·6	
Lipids (% total kCal day^{-1})	34·4 (28·9–42·2)	21·1 (4·3–45·5)	$P < 0·001$
	34·8 ± 1·1	22·6 ± 1·3	
Carbohydrate/sugars (% total kCal day^{-1})	45 (37·9–55)	28·1 (14·8–53·4)	$P < 0·001$
	45·2 ± 1·2	29·8 ± 1·4	
Non-alcoholic kCal day^{-1}	2318 (1403–4104)	1654 (378–4274)	$P = 0·016$
	2303 ± 192	1719 ± 116	
Alcohol kCal day^{-1}	82 (0–287)	849 (120–4106)	$P < 0·001$
	104 ± 26	1027 ± 113	
Alcohol kCal day^{-1} kg^{-1}	1·14 (0–3·56)	12·38 (2·7–51·98)	$P < 0·001$
	1·36 ± 0·31	14·34 ± 1·49	
Alcohol/BMI	ND	33·8 (10·6–78·4)	ND
		39·04 ± 2·55	

ND, not done; NS, not significant.

Table 2 Protein levels of active ghrelin and expression of ghrelin RNA in the study groups

	Control group ($n = 17$) Mean ± SEM	Alcoholic group ($n = 27$) Mean ± SEM	*P*-value
Duodenal ghrelin protein levels (pg mg^{-1} protein)	30 ± 9	56·2 ± 11	NS
Fundic ghrelin mRNA levels	2·45 ± 0·37	3·5 ± 0·753	NS
Duodenal ghrelin mRNA levels	0·183 ± 0·027	0·271 ± 0·056	NS

NS, not significant.

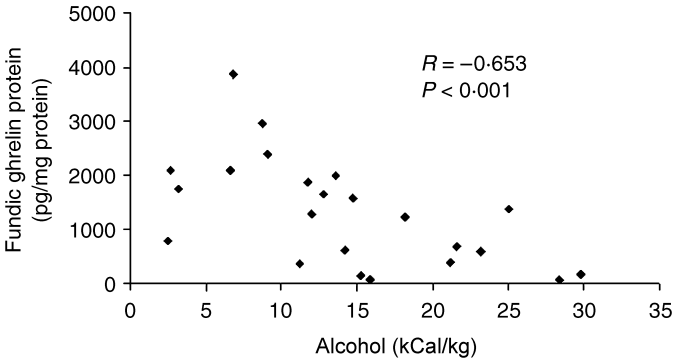

Figure 2 Relationship between alcohol consumption normalized to body weight and fundic ghrelin protein levels. A significant correlation was found between active ghrelin protein levels in fundic biopsies and alcohol consumption suggesting that alcohol interferes with elaboration of ghrelin in the fundus of the stomach.

Ghrelin mRNA levels

No significant difference in ghrelin mRNA levels between alcoholic patients and the control group was observed in fundic and duodenal biopsies. As with ghrelin protein levels, ghrelin mRNA expression was low in duodenal biopsies compared with fundic ones (Table 2). These observations suggest that alcohol does not affect transcription of the ghrelin gene in the stomach or the duodenum.

Correlations

For reasons indicated above, we normalized each patient's mean alcohol consumption to his/her body weight and BMI before investigating correlations. By doing so, an inverse, statistically significant correlation between alcohol consumption normalized to body weight (Fig. 2) and to BMI and fundic ghrelin protein levels was found ($r = –0·677$, $P = 0·005$). By contrast, only a weak, statistically non-significant correlation between alcohol intake and plasma ghrelin levels was observed ($r = 0·2782$, $P = 0·27$). No correlations were observed between alcohol consumption and fundic ghrelin mRNA or duodenal ghrelin protein and mRNA levels.

Discussion

Our study shows that plasma levels of the biologically active form of ghrelin, which is octanoylated on serine three, are reduced in chronic alcoholics who still drank very actively within 24 h prior to determination compared with healthy controls. A similar observation has been published by Addolorato *et al*. in a recent paper [3]. However, not all investigators have found decreased ghrelin levels in alcoholics [8]. There is evidence that the moment of determination of ghrelin might be important and could explain differences between studies for plasma ghrelin levels that tend to increase with alcohol abstinence. It has been shown that ghrelin levels are higher in prolonged alcohol abstainers compared to active drinkers and a positive association with the duration of abstinence has been suggested [10]. In addition, gender-related differences as well as differences related to the subtypes of patients included may account for discordant results between the studies [18,19]. We measured the biologically active form of ghrelin which is octanoylated on serine three. This octanoylated form of ghrelin might be a better index to test the relationship between ghrelin levels and alcohol dependence [3,20,21].

In addition we observed a strong trend towards decreased fundic ghrelin protein levels in chronic alcoholics without, however, affecting expression of mRNA ghrelin levels in fundic biopsies. These observations suggest that alcohol does not affect transcription of the ghrelin genes in fundic biopsies and that post-transcriptional regulatory mechanisms should account for the decrease in plasma and fundic protein levels. Although ghrelin protein levels increased in duodenal biopsies, it was insufficient to compensate for the decrease of ghrelin protein in fundic biopsies. It is unlikely that duodenal ghrelin will be able to compensate for a strong decrease in fundic ghrelin production because the contribution of the duodenum to overall ghrelin levels seems rather low.

Ghrelin seems to increase food intake and reduce the energy expenditure [22]. Alcoholism is a frequent cause for malnutrition [23,24]. Subsequently, lower ghrelin levels in alcoholic patients could be related to the alteration of the nutritional status and energy balance [3]. Therefore, it seems important to take into account the nutritional status of alcoholic patients when examining the relationship between ghrelin levels and alcohol consumption. Thus, in our study, alcohol consumption was expressed in kCal per day and was normalized to body weight and BMI. By doing so, we found a strong inverse correlation between alcohol consumption normalized to body weight or body mass index and fundic ghrelin levels. These observations suggest that alcohol intake does directly affect elaboration of the ghrelin protein in the stomach. However, only a weak trend towards an inverse correlation of alcohol consumption with plasma ghrelin levels was found in our study. This may reflect that other sites of ghrelin secretion that contribute to plasma ghrelin levels, as shown above for the duodenum, do not respond in a similar manner to important alcohol intake. It could also be due to the relatively low number of patients included in the study.

Earlier studies have shown controversial data concerning the relationship between plasma ghrelin levels and craving. Kraus *et al*. [8] did not find any relationship between both phenomena. By contrast, recent investigations suggest that ghrelin could be implicated in the neurobiological mechanisms of alcohol craving. Indeed, Addolorato *et al*. [3] showed a significant positive

correlation between ghrelin levels and craving evaluated by the Obsessive Compulsive Drinking Scale (OCDS) in alcoholic patients where ghrelin was particularly correlated with the compulsive component of the OCDS score. The same study showed a significant association between higher plasma ghrelin levels and higher alcohol craving scores in alcoholic patients. Recently, a link between compulsion and ghrelin was confirmed in animal studies and in alcoholics [17,25]. Our study was not designed to investigate the relationship between craving and plasma ghrelin levels but to evaluate the effect of alcoholism on plasma ghrelin levels and ghrelin production by the stomach. However, our observation that ghrelin levels were decreased both in the plasma and in its main site of secretion, the fundus, is in keeping with the observations of Addolorato *et al*. [3]. It therefore supports the necessity for further investigations in a larger number of patients regarding the connection between ghrelin and craving. In addition, it seems worthwhile to study the relationship between ghrelin and other feeding-related peptides that have been reported to be modified in alcoholism such as for example leptin or insulin [18,26,27]. These studies could help to define more precisely the role of ghrelin in the neurobiological mechanisms of craving.

In conclusion, our data show decreased plasma and fundic ghrelin protein levels in alcoholic patients at the beginning of alcohol withdrawal. Furthermore, reduced ghrelin levels seem to be related to post-transcriptional regulatory mechanisms in the context of alcohol abuse. On the background of our findings, it seems worthwhile to further investigate whether low ghrelin levels might play a significant role in the complex neurobiological mechanisms associated with the appetite regulating network and craving during alcohol dependence.

Address

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