Acute intravenous acyl ghrelin infusion induces thirst but does not affect sodium excretion: two randomized, double-blind, placebo-controlled crossover studies in hypopituitary patients

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Abstract

Objective: Acyl ghrelin, which is the endogenous ligand for the growth hormone secretagogue receptor, potently stimulates pituitary growth hormone release, and to some degree adrenocorticotrophic hormone and prolactin. Ghrelin is also orexigenic and has recently been shown to stimulate renal sodium absorption in rodent models. Increased thirst sensation has been observed as a side effect of acyl ghrelin administration in some human studies. The objective of this clinical trial was to investigate the direct effects of acyl ghrelin on thirst sensation and sodium excretion in hypopituitary patients.

Design: Hypopituitary patients on stable replacement with hydrocortisone and growth hormone were investigated in two double-blind and placebo-controlled crossover studies. The patients received a 5-h intravenous infusion of acyl ghrelin (5 pmol/kg/min in the first study and 1 pmol/kg/min in the second study). Thirst sensation was measured on a Visual Analog Scale (VAS). In the second study plasma osmolality, vasopressin, copeptin, water intake, diuresis and urinary excretion of sodium and creatinine were measured.

Results: In the initial study, acyl ghrelin (5 pmol/kg/min) increased thirst sensation (time × treatment analysis of variance for the effect of acyl ghrelin infusion $P = 0.003$). In the second study acyl ghrelin (1 pmol/kg/min) also increased thirst ($P = 0.04$) but did not affect urinary excretion of either sodium or water.

Conclusions: We demonstrate that acyl ghrelin infusion increases thirst sensation, without affecting sodium excretion or diuresis in human subjects.

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Introduction

Acyl ghrelin (AG) is the endogenous agonist for the growth hormone secretagogue receptor (GHS-R) (1). Activation of the GHS-R in the hypothalamus and the pituitary gland stimulates the secretion of growth hormone (GH), adrenocorticotrophic hormone (ACTH) and prolactin (2, 3).

AG also controls energy homeostasis by inducing appetite (4) and it fluctuates in a distinct pattern with pre-prandial elevations followed by postprandial troughs (5).

Feeding and drinking are usually closely connected (6) and increasing evidence suggests that AG also

affects fluid intake, vasopressin secretion and diuresis. As an example, mRNA of the GHS-R is detected in the circumventricular organ (the subfornical organ (SFO)) and its neurons are depolarized by AG exposure (7). The SFO is implicated in thirst sensation (8, 9) and in an eel model, intraventricular AG administration dose-dependently decreases water intake (10). AG is also antidipsogenic in polyethylene glycol-treated hypovolemic rats (11) and in rats stimulated by angiotensin II or hypertonic saline (12). Activation of the GHS-R potentially stimulates vasopressin release both *in vitro* (13), and *in vivo*, in rodents (14).

Clinical effects of AG on thirst or vasopressin secretion have not yet been investigated, but increased thirst or 'dry mouth' was registered as an adverse event in 2.5% (8 of 320) of volunteers participating in clinical studies involving AG infusions (15). Only one clinical study addressed acute renal effects of AG, showing no acute effects on water or sodium excretion (16).

It remains, however, difficult to dissect the direct effects of AG from secondary effects related to AG-induced GH and cortisol release, because GH causes sodium and water retention (17, 18).

In two double-blind, placebo-controlled and randomized experiments in hypopituitary patients on stable replacement therapy with GH and hydrocortisone, we first assessed whether AG infusion-induced thirst (primary endpoint) and secondly, we investigated the effects of AG on water intake, diuresis and sodium excretion rate.

Subjects and methods

The studies were conducted in accordance with the Helsinki Declaration and all subjects gave their oral and written informed consent to participate. The study protocols were approved by the Central Region Denmark Ethics Committee and the Danish Medicines Agency. The protocols were registered at Clinicaltrials.gov NCT00139945 (Study 1) and NCT01209416 (Study 2) before the onset of enrolment. The Good Clinical Practice (GCP) Unit of Aarhus University Hospital approved Study 1. The patients also participated in a metabolic study and data on metabolism have been previously been reported (19, 20).

Preparation of synthetic ghrelin

Synthetic human AG (Study 1: human acyl ghrelin, NeoMPS, Strasbourg, France. Study 2: GMP-grade human

AG; Bachem, Weil am Rhein, Germany) was dissolved in isotonic saline. All procedures complied with GDP and GCP guidelines.

Subjects

Both studies each included eight hypopituitary men on stable replacement therapy with GH and hydrocortisone for >3 months. Three patients from Study 1 also participated in Study 2. None of the patients had diabetes, hypertension, renal disease or any other concomitant chronic disease. The participants of Study 1 were 53 ± 4 years of age and had a BMI of $31.6 \pm 1.0 \text{ kg/m}^2$. In Study 2, the participants were 53 ± 5 years of age and had a BMI of $30.3 \pm 4.6 \text{ kg/m}^2$.

Study protocols

All participants were examined on two occasions separated by a minimum of 2 weeks. The studies were performed in a quiet, thermoneutral indoor environment. The subjects fasted during the trials, but were allowed oral water intake *ad libitum*. Fresh and cool tap water was available at all times at the bedside for all subjects during both studies and both study days. Tap water was provided in a jug from which, water was served in a clear glass with a straw, which made it easier to drink. Each jug of tap water contained 1.25 L and water consumption was measured as a multiple of 1.25 L (depending on the number of jug servings) minus the remaining water in the jug and glass. Water intake was measured in study 2. The patients emptied their bladder before starting the research interventions. The interventions commenced at 0800h after an overnight fast. In two double-blind and placebo-controlled crossover studies, each subject underwent two randomized and double-blinded interventions. One i.v. cannula was inserted into the antecubital region for infusion, and one i.v. cannula was positioned in a dorsal hand vein for blood sampling. The latter was placed in a heated box at 65 °C (Study 1) or in a heat pad (Study 2) for sampling of arterialized blood. Randomization procedure was performed by the pharmaceutical services by a computerized algorithm.

After observing an effect of AG on thirst in study 1, a 5-fold lower and more physiological dose of AG was used in study 2. Previous experiments demonstrated that an infusion rate of 1 pmol/min/kg also exerted endocrine effects (21).

At $t=0$, AG (Study 1: 5 pmol/kg/min. Study 2: 1 pmol/kg/min) or placebo (isotonic saline 'sal') was

commenced. The sodium load was equal on each occasion. The infusion periods lasted 300 min.

Thirst sensation was determined by a Visual Analog Scale (VAS) at $t=0$, 120 and 300 min in study 1 and at $t=0$, 60, 120, 180, 240 and 300 min in study 2. Subjects were instructed to make a single vertical mark on a horizontal line (possible scores 0–100 mm) to indicate their current sensation between 0 corresponding to ‘not thirsty at all’ and 100 mm corresponding to ‘as thirsty as you could imagine’. A blinded observer measured the scores.

In study 2, the patients emptied their bladder at $t=120$ and 300 min and the urine was measured by volume.

Plasma concentrations of arginine-vasopressin (AVP) was measured in Study 2 at $t=0$, 120, 180 and 300 min. Serum concentrations of copeptin was measured at $t=0$, 120 and 300 min, and plasma osmolality was measured at $t=0$, 120 and 300 min in study 2.

A hyperinsulinemic–euglycemic clamp (insulin 0.6 mU/kg/min, Actrapid, Novo Nordisk) was performed from $t=120$ to 300 min. Plasma glucose was clamped at 5.0 mmol/L by adjusting the infusion rate of 20% glucose according to plasma glucose measurements every 10 min. The period from $t=0$ to 120 min is referred to as the basal period and the period from $t=120$ to 300 min as the clamp period.

Biochemical analyses

Urine was stored and frozen at -20°C until analyses. Sodium was measured by ISE potentiometry and creatinine was analyzed using CREP2 on a Cobas 6000 analyzer (Roche Diagnostics).

AVP was measured in plasma after extraction in a Sep-Pak Plus C18 cartridge (Waters, Milford, MA, USA) using a previously described radioimmunoassay (RIA) (22). The specific AVP antibody (AB3096, produced by P. Bie, Department of Physiology and Pharmacology, University of Southern Denmark) was incorporated in the assay (23) with a detection limit of 0.10 pg/mL.

Serum was stored and frozen at -80°C until analyses. Serum concentrations of copeptin was measured by time-resolved amplified cryptate emission technique on the B-R-A-H-M-S KRYPTOR Compact Plus system (Thermo Fisher Scientific).

Plasma osmolality was determined using the freezing point depression method (Osmometer 3900, Advanced Instruments).

Plasma glucose was analyzed bedside using the glucose oxidase method (YSI 2300 STAT Plus; Yellow Springs Instruments, Yellow Springs, OH, USA).

Statistical analysis

Results are expressed as mean and standard errors (S.E.) and as median and 25/75 percentiles (AVP). VAS was analyzed by two-way ANOVA for repeated measurements. The interaction between time and treatment (‘time $\times$ treatment’) was considered the term of interest. Student’s two-tailed paired t test was used to analyze the effect of AG and sal on urinary excretions, volumes and fluid intake. P values less than 0.05 were considered significant. Statistical analysis was performed using SigmaPlot for Windows Version 11.0, (Systat Software, CA, USA).

Results

Ghrelin response

Ghrelin concentrations increased from 483 ± 52 pg/mL to 5124 ± 421 pg/mL (total levels) in Study 1 (19) and from 49.6 ± 9.4 pg/mL at baseline to 1015.1 ± 34.8 pg/mL (acylated ghrelin levels) in Study 2 (20) in response to AG infusion.

Study 1

VAS: Baseline VAS was similar on both study days (3.0 ± 0.7 mm (AG) vs 3.2 ± 0.7 mm (sal), $P=0.73$). Overall, there was a time $\times$ treatment effect of AG infusion, ANOVA- $P=0.003$. VAS score increased during AG infusion at $t=120$ min (7.3 ± 0.4 mm (AG) vs 3.8 ± 0.7 mm (sal), $P=0.004$) and remained elevated at $t=300$ min (5.9 ± 0.5 mm (AG) vs 3.0 ± 1.1 mm (sal), $P=0.006$) (Fig. 1A).

Study 2

VAS: Baseline VAS was similar on both study days (2.6 ± 0.7 mm (AG) vs 2.2 ± 1.0 mm (sal), $P=0.67$). Overall, there was no time $\times$ treatment interaction of AG, ANOVA- $P=0.13$, but a paired t test revealed a significant increase of VAS score during AG infusion at $t=120$ min, $P=0.04$ (Fig. 1B).

Fluid intake and diuresis: Oral water intake was similar in response to AG and saline infusion (529 ± 82 mL (AG) vs 467 ± 75 mL (sal), $P=0.39$) and the total amount of oral water intake and i.v. infusion was also similar during both study days (1207 ± 85 mL (AG) vs 1179 ± 79 mL (sal), $P=0.73$). The diuresis was similar in the basal period (77 ± 20 mL/h (AG) vs 77 ± 14 mL/h (sal), $P=0.97$) and increased in response to i.v. fluid load during the clamp,

Figure 1

Thirst and fluid intake during acyl ghrelin and saline infusion. (A and B) Thirst score increased in response to AG infusion in both study 1 (A) and 2 (B). (C) Oral water intake was equal on the two study days with AG and saline infusion in study 2. (D) The total fluid intake which includes both oral water intake and intravenous infusions was also similar on both study days in study 2.

but the increase was similar on the two study days (152 ± 33 mL/h (AG) vs 173 ± 18 mL/h (sal), $P = 0.54$). We examined if AG caused fluid retention by comparing the difference in total fluid load and diuresis, but AG did not affect net fluid balance (479 ± 153 mL (AG) vs 393 ± 81 mL (sal), $P = 0.58$) (Figs 1C, D and 2A, B).

Urinary sodium, creatinine and sodium-to-creatinine ratio: Urinary excretion of sodium ($\mu\text{mol/kg/h}$) was similar in both the basal (95 ± 19 (AG) vs 94 ± 13 (sal), $P = 0.94$) and the clamp period (114 ± 17 (AG) vs 144 ± 17 (sal), $P = 0.27$), and the excretion of creatinine was also similar in the basal period (in $\mu\text{mol/kg/h}$) (6.3 ± 0.6 (AG) vs 6.3 ± 0.7 (sal), $P = 0.93$) and the clamp period (7.5 ± 0.4 (AG) vs 7.6 ± 0.7 (sal), $P = 0.83$). Urinary sodium excretion was normalized to urinary creatinine excretion by calculating the sodium/creatinine ratio in both the basal and the clamp periods, respectively. Normalized urinary sodium was similar in the basal period (15.7 ± 3.1 (AG) vs 15.2 ± 1.5 (sal), $P = 0.88$) and in the clamp period (15.3 ± 2.2 (AG) vs 19.3 ± 2.7 (sal), $P = 0.17$) (Fig. 3A, B and C).

Plasma arginine-vasopressin, serum copeptin and plasma osmolality: Arginine-vasopressin was similar at baseline (in pg/mL) (0.21 ($0.20/0.52$) (AG) vs 0.19 ($0.14/0.52$) (sal),

Figure 2

Diuresis and fluid balance during acyl ghrelin and saline infusion in study 2. (A) Diuresis was similar during AG and saline infusion in the basal period and in the clamp period. (B) There was no difference in fluid balance as estimated by the difference in total fluid intake and diuresis on the two study days.

$P = 0.16$), and there was no significant time $\times$ treatment effect, ANOVA- $P = 0.09$ (Fig. 4A, B and C). By chance, there was a slight difference in serum copeptin at baseline before AG and sal infusion between study days (in pmol/L) (3.5 ± 0.5 (AG) vs 3.1 ± 0.5 (sal), $P = 0.04$), but no time $\times$ treatment effect, ANOVA- $P = 0.91$. Plasma osmolality was similar at baseline (in mosm/kg) (279.9 ± 1.2 vs 280.3 ± 1.4 , $P = 0.77$), and there was no time $\times$ treatment effect, ANOVA- $P = 0.76$. Plasma osmolality correlated positively with plasma arginine-vasopressin during saline infusion (Fig. 5), whereas no association was recorded during AG infusion.

Figure 3

Urine excretion of sodium and creatinine during AG and saline infusion in study 2. Acyl ghrelin did not have an impact on sodium excretion (A), creatinine excretion (B) or the sodium-to-creatinine ratio (C) during the basal or the clamp periods.

Figure 4

Plasma vasopressin and osmolality during acyl ghrelin and saline infusion in study 2. AG did not affect plasma vasopressin (A), serum copeptin (B) or plasma osmolality (C) as compared to saline infusion.

Figure 5

Correlation analyses of plasma osmolality and vasopressin concentration during acyl ghrelin and saline infusion in study 2. During saline infusion, there was a positive association between osmolality and vasopressin, but the association was abrogated by AG infusion.

Discussion

The aim of the present study was to investigate the effects of intravenous AG infusion on thirst, water intake, diuresis and sodium excretion rate in human subjects. The inclusion of patients with hypopituitarism allowed us to investigate GH- and ACTH-independent effects of AG. We document for the first time that intravenous AG infusion acutely induces thirst, but does not affect plasma vasopressin, serum copeptin, water intake, diuresis or sodium excretion in humans.

Eating and drinking are temporally closely connected and the amount of water intake is associated with the size of a meal (6). AG possesses powerful orexigenic actions (4, 5), but it has not been established if AG also stimulates thirst. Healthy subjects participating in our earlier blinded AG infusion studies focusing on metabolic effects and pharmacokinetics of AG reported increased thirst during some of the study days. Thirst has not previously been studied as an endpoint in clinical studies, but has only been recorded as a side effect in some trials (15). This prompted us to perform a pilot study (Study 1) in which we measured thirst at baseline and after 2 and 5 h of AG infusion in hypopituitary patients (19). We clearly documented that AG infusion induced thirst independently of GH and ACTH. In Study 2, we used a lower AG dose and also measured electrolyte and fluid balances and systemic AVP and copeptin levels. The effect

of the lower AG dose on thirst in Study 2 was smaller and suggests a dose-dependent effect of AG. AG did not impact significantly on systemic arginine-vasopressin, copeptin, plasma osmolality or blood pressure. Ghrelin infusion abrogated the positive association between plasma vasopressin and osmolality suggesting that ghrelin-induced thirst is vasopressin independent. Traditionally, thirst has been considered to evolve in response to osmolality and fluid changes, but recently, it has been documented that thirst sensation precedes these changes and the neuronal mechanisms have been unraveled (9). We hypothesize that AG could play a role in these molecular mechanisms, but this remains to be investigated.

A few studies have investigated the possible dipsogenic effects of AG in animals (10, 11, 12). The protocols of the published studies in animal models are not directly comparable with our clinical protocols, because they address the dipsogenic effect of AG after interventions, which are usually associated with increased thirst *per se* for example water deprivation, pharmacologically induced hypovolemia, hypertonic saline infusion and angiotensin II injection. In rats, AG exposure had no dipsogenic effects (12), which is in line with our observations in humans. Mietlicki *et al.* studied the effect of AG on water intake during different dipsogenic conditions in rats and observed that AG decreased water intake after exposure to hypertonic saline and angiotensin II alone and in combination (12). Hashimoto *et al.* also report anti-dipsogenic effects of AG in rats after hypovolemia and angiotensin II injections (11). The reason for this apparent species difference remains to be studied.

AG administration potentially induces GH secretion and to a lesser extent also ACTH and prolactin release (3), but the effect of AG on vasopressin in humans has, to our knowledge, not been investigated previously (15). Animal models and *in vitro* experiments suggest that AG and GHS-R stimulate vasopressin secretion (13). AG also activates hypothalamic neuropeptide Y (NPY) (24, 25) that has been shown to induce vasopressin release in rats (26). Our data contrast with *in vitro* experiments and animal models, because we did not record effects of AG on systemic vasopressin levels in humans.

AG administration causes hypotension and decreases sympathetic activity in healthy subjects (27) and decreases arterial pressure, but does not change diuresis or sodium excretion in patients with chronic heart failure (16). Several peripheral tissues including the kidney express GHS-R mRNA (28) and bind synthetic GHS (29). AG is produced at the basolateral membrane in the distal tubular

cells and in the collecting ducts (30), which are important for electrolyte homeostasis and systemic blood pressure. The GHS-R is localized on the cortical collecting duct, and intra-renal infusion of AG decreases arterial pressure and sodium excretion when compared to baseline levels without affecting the glomerular filtration rate (GFR) (28). The GHS-R co-localizes with the epithelial sodium channel $E_{Na}C$ in rat kidney (31) and intra-renal AG infusion signals through adenylyl cyclase, cAMP, protein kinase A and causes increased apical $E_{Na}C$ expression and antinatriuresis (31). $E_{Na}C$ -mediated sodium reabsorption depends on proteinuria and as rodents excrete considerable amounts of protein in their urine (32), species-specific differences between humans and rats may exist regarding the effect of AG on natriuresis.

Systemic AG levels increase even by low-dose subcutaneous administration (33), and it remains a possibility that intra-renal AG infusions also results in elevated systemic AG levels and effects. Of note, sodium retention is a well-known effect of GH administration in rats (34) and humans (18) involving increased activity of the Na^+ , K^+ , $2Cl^-$ cotransporter in the medullary thick ascending limb of the kidney (35). To obviate the AG-induced GH and ACTH secretion, hypopituitary patients constitute an attractive *in vivo* model for studying GH- and cortisol-independent effects of AG. The natriuretic effect of the AG antagonist [d-Lys-3]-GHRP-6 in the study by Kemp *et al.* strongly points toward AG as a physiological important regulator of sodium excretion in rodents (31), but our study indicates that AG *per se* does not play a significant role in diuresis or sodium excretion rate in humans.

The strength of our study is that the design allows investigation of the direct effects of AG, but certain limitations also apply. First, AG levels were elevated above the physiological range. Second, observations of thirst sensation in fasting subjects in the postabsorptive state may not apply to non-fasting conditions. Third, the sample size was quite small and for the insignificant results, a type II error cannot be ruled out. Fourth, lack of water intake assessment in all subjects during the fasting period from the evening before the infusion study and during the interventions in Study 1 is also a limitation in the interpretation of the results. Finally, it should be added that one patient in Study 1 had central diabetes insipidus and was on stable replacement with desmopressin.

In conclusion, AG administration increases thirst in hypopituitary subjects without affecting water intake, diuresis or sodium excretion. The physiological and clinical implications remain to be further characterized.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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