



Ghrelin levels are reduced in Rett syndrome patients with eating difficulties

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ABSTRACT

Most patients with Rett syndrome (RTT) have both gastrointestinal problems and somatic growth failure, including microcephaly. Ghrelin is a peptide hormone involved in growth hormone secretion, interdigestive motility, and feeding behavior. Plasma ghrelin assays have previously been described for other neurodevelopmental disorders. To examine the pathophysiology of RTT, we measured plasma levels of ghrelin in patients with RTT. A case-control study examining plasma levels of ghrelin, serum growth hormone, and insulin-like growth factor-1 (IGF-1) was performed on 27 patients with RTT and 53 controls. Plasma levels of total (T)- and octanoyl (O)-ghrelin were significantly lower in patients with RTT than in controls. Plasma levels of T-ghrelin correlated significantly with serum IGF-1 levels and head circumference. Significantly lower levels of plasma T-ghrelin and O-ghrelin were observed in RTT patients with eating difficulties, while lower levels of plasma T-ghrelin were observed in RTT patients with constipation, in comparison to patients without either of these symptoms. Alterations in plasma ghrelin levels may reflect various clinical symptoms and signs in RTT patients, including growth failure, acquired microcephalus, autonomic nerve dysfunction, and feeding difficulties. We describe the role of ghrelin in RTT and suggest this peptide as a novel biological marker in patients with RTT.

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1. Introduction

Rett syndrome (RTT, MIM 312750) is an X-linked neurodevelopmental disorder, caused in the vast majority of cases by mutations in *Methyl-CpG-binding protein 2 (MeCP2)* (Amir et al., 1999). RTT is characterized by deceleration of head growth, mental retardation, motor disabilities, autistic behavior, epilepsy, periodic breathing, coldness of extremities, eating difficulties, constipation, emotional disturbances, and sleep disruption, followed by somatic growth failure (Oddy et al., 2007; Percy et al., 2010; Schultz

et al., 1993). Ghrelin is an acylated peptide hormone produced mainly in gastrointestinal (GI) tissues (Kojima et al., 1999). Ghrelin exerts multiple physiological functions including the stimulation of growth hormone (GH) secretion, the modulation of energy metabolism and circulation, and the regulation of autonomic functions and seizure threshold (Kojima and Kangawa, 2005). The exogenous administration of ghrelin stimulates GH secretion and increases caloric intake and GI motility. Symptoms and signs of RTT, such as growth failure, GI problems, autonomic dysfunction, and neuropsychiatric symptoms, seem to be explained by the disturbance in ghrelin secretion. Therefore, we have speculated that ghrelin plays an important role in patients with RTT. However, plasma ghrelin has not been studied previously in RTT. We compared the plasma levels of ghrelin in RTT patients and healthy controls and assessed the relationship between plasma ghrelin, growth factors, and clinical symptoms and signs.

2. Methods

Clinical diagnosis of RTT was confirmed in 27 female patients according to the recently proposed Rett Syndrome Diagnostic Criteria (Percy et al., 2010). We

Abbreviations: RTT, Rett syndrome; GH, growth hormone; IGF-1, insulin-like growth factor-1; T-ghrelin, total-ghrelin; O-ghrelin, octanoyl-ghrelin; MeCP2, methyl-CpG-binding protein 2; GI, gastrointestinal; DQ, developmental quotient; IQ, intelligence quotient; BMI, body mass index; OFc, occipito-frontal head circumference; EDTA-2Na, disodium dihydrogen ethylenediamine tetraacetate dihydrate; RIA, radioimmunoassay; IRMA, immunoradiometric assay; SD, standard deviations; CNS, central nervous system; SNpc, substantia nigra pars compacta.

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Table 1
Clinical characteristics of patients with Rett syndrome.

Characteristics	Controls			RTT						p	
				Correlations				Correlations			
				T-ghrelin	O-ghrelin			T-ghrelin	O-ghrelin		
Age (years)	16.9	(±10.2)	[N=53]	0.30*	0.34*	15.8	(±8.3)	[N=27]	−0.40*	−0.36	0.60
Weight (kg)	39.8	(±16.1)	[N=53]	0.00	0.05	27.9	(±11.7)	[N=27]	−0.59**	−0.48*	0.00**
BMI (kg/m ²)	18.7	(±3.3)	[N=53]	−0.13	−0.16	15.4	(±3.5)	[N=27]	−0.55**	−0.31	0.00**
BMI-Z	−0.1	(±1.0)	[N=53]	−0.59**	−0.58**	−2.2	(±2.2)	[N=27]	−0.21	0.07	0.00†
Height/length (cm)	141.8	(±23.6)	[N=53]	−0.01	0.11	131.8	(±19.3)	[N=27]	−0.49**	−0.57**	0.06
Height/length-Z	−0.3	(±0.9)	[N=53]	−0.36**	−0.22	−2.7	(±0.9)	[N=27]	0.00	0.06	0.00**
OFC (cm)	52.1	(±3.1)	[N=27]	−0.74**	−0.56**	50.4	(±2.5)	[N=27]	−0.66**	−0.49**	0.03*
OFC-Z	1.9	(±1.5)	[N=27]	−0.61**	−0.53**	0.3	(±1.8)	[N=27]	−0.66**	−0.48*	0.00**
Total-ghrelin (fmol/ml)	215.5	(±115.0)	[N=53]	−	0.81**	113.5	(±51.9)	[N=27]	−	0.67*	0.00†
Octanoyl-ghrelin (fmol/ml)	26.8	(±14.1)	[N=53]	0.81**	−	16.9	(±8.3)	[N=27]	0.67**	−	0.00**
O/T ratio	0.13	(±0.05)	[N=53]	−0.33†	0.20	0.16	(±0.1)	[N=27]	−0.39*	0.34	0.06

All of the data represent means ± standard deviations. BMI-Z, BMI-for-age Z score; Height/length-Z, height/length-for-age Z score; OFC, occipito-frontal head circumference; OFC-Z, OFC-for-age Z score; T-ghrelin, total ghrelin; O-ghrelin, octanoyl ghrelin. O-ghrelin was significantly lower in patients with RTT than in controls (** $p \leq 0.01$). Weight, BMI, height/length ratio, and OFC-Z in RTT was significantly lower in comparison to controls (** $p \leq 0.01$). Age, weight, and OFC-Z demonstrated significant inverse correlations with the plasma levels of T-, and O-ghrelin in both RTT patients and controls.

* $p \leq 0.05$.

** $p \leq 0.01$.

measured plasma levels of ghrelin in 27 patients with RTT and 53 age- and gender-matched healthy controls. A genetic analysis of *MeCP2* was performed in all patients with RTT. Of the 27 RTT patients, 9 were in stage III and 18 were in stage IV. All of the RTT patients had a developmental quotient (DQ) or intelligence quotient (IQ) below 20. None of the RTT patients had undergone gastrostomy or had received medications targeting the autonomic nervous system, which might have influenced the plasma ghrelin levels. Eating difficulties were defined as present if dietary intake required more than 30 min, on average, during each mealtime in the 2 weeks prior to this study, as recorded by both caretakers and occupational therapists (Oddy et al., 2007). The clinical data that we collected on RTT patients included weight, height, body mass index (BMI), and occipito-frontal head circumference (OFC). These data were converted into the standard deviation Z score established by the US National Center for Health Statistics/World Health Organization (de Oris et al., 2006). Written informed consent was obtained from each patient's or control's parents. The study protocol was approved by the Ethical Committee of the Kurume University School of Medicine.

2.1. Extraction and derivation

The extraction of plasma ghrelin from blood was performed by a method described previously (Hosoda et al., 2000). In brief, samples were obtained by venipuncture between 0800 and 1000 after an overnight fast. Blood samples were put into chilled polypropylene tubes containing EDTA-2Na and aprotinin (700 kIU/mL), and immediately centrifuged to obtain plasma samples. The separated plasma samples were acidified by the addition of 1.0 N HCl (10% of sample volume), and then stored at -80°C . The plasma samples were semi-purified before the ghrelin radioimmunoassay (RIA) using Sep-Pak C18 cartridges. Two ghrelin-specific RIAs were used; one recognizes the N-terminal portion of octanoyl-modified active ghrelin (O-ghrelin), while the other recognizes the C-terminal portion of ghrelin irrespective of its octanoyl-modification. The plasma levels of ghrelin were determined by a validated in-house RIA as previously described (Hosoda et al., 2000). O-ghrelin is post-translationally octanoylated at Ser3 (Hosoda et al., 2000; Kojima et al., 1999). Plasma levels of total ghrelin (T-ghrelin) were estimated as the sum of non-octanoyl and octanoyl ghrelin levels. Both antibodies used in our ghrelin-specific RIAs exhibited complete cross-reactivity with human n-octanoyl ghrelin (Hosoda et al., 2000). The intra- and inter-assay coefficients of variations were all less than 7.0%. The detection limits of plasma T- and O-ghrelin were 40 and 5 fmol/mL, respectively. All assays were performed in duplicate.

2.2. Growth hormone (GH) and insulin-like growth factor-1 (IGF-1) assays

Serum concentrations of GH and IGF-1 were measured in duplicate by immunoradiometric assays according to the manufacturer's protocol (Active Growth Hormone IRMA DSL-1900 and Active Non-Extraction IGF-1 IRMA DSL-2800, respectively, Diagnostics System Laboratories, Webster, TX, USA) or by radioimmunoassay kit (SRL Inc., Tokyo, Japan). Each assay was calibrated with manufacturer-supplied standards.

2.3. Statistical methods

The concentrations of plasma ghrelin and serum GH and IGF-1 were compared both between and within groups by *t*-tests, and Pearson's correlation coefficients

were used to measure monotonic associations between variables. Data are summarized as means ± standard deviations (SD). For statistical analysis, we used analysis of variance. The reference curves for plasma ghrelin concentrations were constructed by additive models incorporating the interaction terms of age and age × disease group as the nonlinear parameters. The approximate significance of smooth terms was obtained by *F*-tests. If a difference deviated by more than 2 SDs from the mean, the data were excluded as outliers. $p \leq 0.05$ was considered significant.

3. Results

3.1. Comparisons between Rett syndrome patients and controls

Plasma levels of T-ghrelin were significantly lower in patients with RTT (113.5 ± 51.9) than in controls (215.5 ± 115.0), at 52.7% of the control values ($p \leq 0.01$). Plasma levels of O-ghrelin were significantly lower (16.9 ± 8.3) than in controls (26.8 ± 14.1), at 63.1% of the control values ($p \leq 0.01$).

3.2. The clinical background variables of Rett syndrome patients and the concentrations of plasma ghrelin

Neither T- nor O-ghrelin levels varied significantly with type of *MeCP2* mutations. Weight, BMI, height, and OFC-Z in RTT patients were significantly lower than in controls ($p \leq 0.01$). Age, weight, and OFC-Z showed significant inverse correlations with plasma levels of T- and O-ghrelin in both RTT patients and controls (Table 1). Moreover OFC-Z showed significantly positive correlations with serum levels of IGF-1. Plasma levels of T-ghrelin correlated significantly with IGF-1 levels (179.4 ± 107.7) in patients with RTT ($p \leq 0.05$). In controls, plasma levels of T- and O-ghrelin tended to decrease until around 10 years of age, then increased during adolescence until they plateaued at around 25 years of age; these patterns were different in patients with RTT (Fig. 1A and B). RTT patients suffered from the following symptoms: periodic breathing (17/27), sleeping disruption (18/27), cold hands/feet (22/27), epilepsy (23/27), eating difficulties (7/27), and constipation (14/27). Plasma levels of T-ghrelin were significantly lower in RTT patients with eating difficulties and constipation than in patients without each symptom ($p \leq 0.01$ and $p \leq 0.05$, respectively). Plasma levels of O-ghrelin were also significantly lower in RTT patients with eating difficulties than in those without ($p \leq 0.05$) (Table 2).

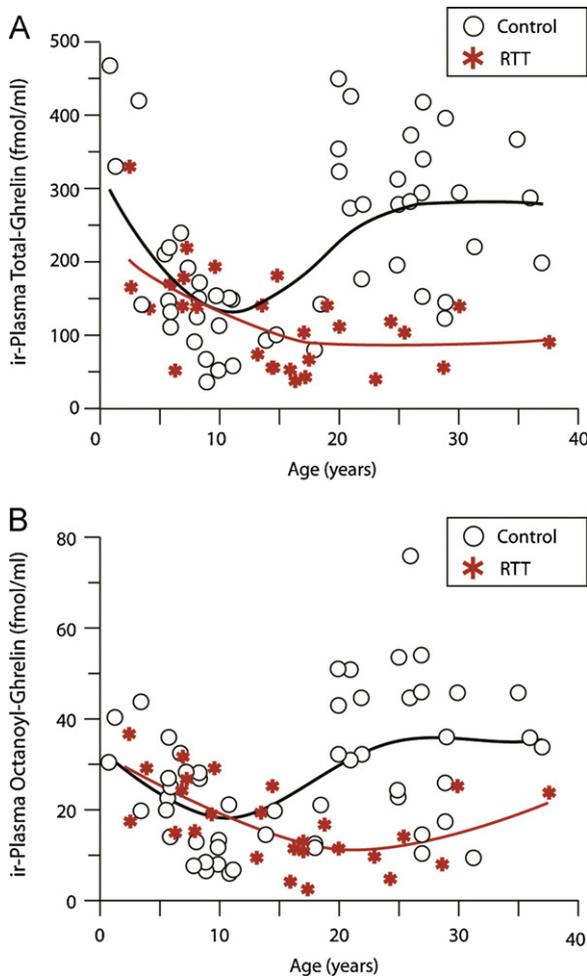


Fig. 1. Reference curves for plasma ghrelin levels were constructed using generalized additive models. The following additive models were fitted: plasma ghrelin concentrations = $s(\text{age}) + \text{disease group} + s(\text{age} \times \text{disease group})$ where $s(\)$ is a smoothing function. The test for the age \times disease group interaction for concentrations of both plasma T-ghrelin (A) and O-ghrelin (B) was statistically significant with p values of 0.004 and 0.01, respectively: T-ghrelin = total ghrelin; O-ghrelin = octanoyl ghrelin.

4. Discussion

4.1. Age and ghrelin

In general, plasma ghrelin levels are high during infancy, then decrease until puberty, at which time they increase in an age-dependent manner (Chanoine, 2005). In this study, patients with

RTT showed a different pattern of ghrelin level variability compared to both the previous report (Chanoine, 2005) and to our controls. Our results showed that the ghrelin levels in RTT continued to decrease after 10 years of age (Fig. 1A and B).

4.2. Ghrelin and signs and symptoms of Rett syndrome

Plasma ghrelin inversely correlated with weight, BMI, and height in controls, whereas these relationships were not observed in RTT (Table 1). Mice with *Mecp2*-null mutations are significantly smaller than wild-type mice (Guy et al., 2001), and numerous genes in the hypothalamus were found to be deregulated in both *Mecp2*-null and *MECP2-Tg* male mice (Ben-Shachar et al., 2009). Interestingly, ghrelin-producing cells exist even in the internuclear space of the hypothalamus, and ghrelin exerts an orexigenic effect via a feeding-related neuropeptide (Ferrini et al., 2009). Ghrelin is also known to promote proliferation of nerve cells and neural stem cells, and it maintains normal plasma IGF-1 levels via GHS-R, thus promoting physical growth (Sun et al., 2004). Previous reports have pointed out an association between autism and plasma and cerebrospinal fluid IGF-1 and OFC (Huppke et al., 2001; Riikonen, 2008). Interestingly, OFC inversely correlated with plasma ghrelin concentrations. This implies that ghrelin may be involved in neurogenesis or neuroprotective effects, either directly or via induction of IGF-1. On the other hand, about two-thirds of circulating ghrelin is derived from the stomach (Kojima and Kangawa, 2005). Although plasma levels of T- and O-ghrelin were lower in RTT than in controls, the ratio of O-ghrelin to T-ghrelin did not differ significantly between the two groups. This suggests that ghrelin production and substrate modification was properly performed in the stomach. The pathophysiology of RTT is still unknown. We demonstrated that plasma ghrelin levels were markedly decreased in patients with RTT. Moreover, they were lower in RTT patients with eating difficulties and constipation. A recent report showed that acetylcholine regulates ghrelin secretion as an interactive modulator of the autonomic nerve system (Hosoda and Kangawa, 2008). These results may reflect dysfunction in the integration of the central nervous system (CNS) and autonomic and peripheral nervous systems in RTT. Therefore, ghrelin may be the biological marker of CNS, autonomic nervous system dysfunction, and growth failure in RTT. Previous neuropathological studies have demonstrated decreased melanin content of the zona compacta nigra and a reduction in the number of basal forebrain cholinergic neurons in the CNS (Armstrong, 1992). Patients with RTT also were reported to have Parkinson-like symptoms, and *Mecp2*-null mice (*Mecp2*^{null/y}) showed dysfunction of the dopaminergic system (Samaco et al., 2009). Jiang et al. (2008) reported that ghrelin’s receptor, GHSR-1a, is highly expressed in the substantia nigra pars compacta (SNpc), and ghrelin inhibited MPTP-induced dopaminergic neuronal loss in the SNpc in a mouse model of Parkinson’s disease.

Table 2
Plasma levels of T- and O-ghrelin in RTT patients by symptom.

Symptoms	Total ghrelin			<i>p</i>	Octanoyl ghrelin			<i>p</i>
	+	–			+	–		
Periodic breathing	104.9 (±57.6) [N=17]	128.2 (±39.0) [N=10]	0.27	17.7 (±8.9) [N=17]	15.6 (±7.3) [N=10]	0.54		
Sleep disruption	111.8 (±57.7) [N=18]	116.9 (±40.9) [N=9]	0.82	17.6 (±8.6) [N=18]	15.6 (±7.8) [N=9]	0.55		
Cold hands/feet	116.6 (±51.1) [N=22]	102.0 (±60.3) [N=5]	0.59	16.7 (±8.4) [N=22]	17.9 (±8.6) [N=5]	0.78		
Epilepsy	107.9 (±53.6) [N=23]	146.1 (±25.2) [N=4]	0.18	17.1 (±8.5) [N=23]	16.1 (±8.3) [N=4]	0.82		
Eating difficulties	56.5 (±20.9) [N=7]	133.5 (±44.0) [N=20]	0.00**	11.5 (±7.5) [N=7]	18.8 (±7.8) [N=20]	0.04*		
Constipation	93.5 (±58.2) [N=14]	135.1 (±34.7) [N=13]	0.03	15.4 (±8.9) [N=14]	18.6 (±7.5) [N=13]	0.33		

All of the data represent means \pm standard deviations. T-ghrelin, total ghrelin; O-ghrelin, octanoyl ghrelin. Significantly lower levels of plasma T-ghrelin were observed in patients with eating difficulties (** $p \leq 0.01$) or constipation (* $p \leq 0.05$).

* $p \leq 0.05$.
** $p \leq 0.01$.

5. Conclusions

Intravenously administered forms of ghrelin have recently become clinically available and have been safely used for the treatment of patients with functional dyspepsia, eating disorders, and overly low BMI. Ghrelin increases daily food intake, enhances the sensation of hunger, and augments growth hormone release (Akamizu et al., 2008). Therefore, our study demonstrated that ghrelin is potentially an effective treatment for various symptoms and signs in patients with RTT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijdevneu.2011.07.003.

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