Analysis of plasma ghrelin in patients with medium-chain acyl-CoA dehydrogenase deficiency and glutaric aciduria type II

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Abstract

Objective: Ghrelin requires a fatty acid modification for binding to the GH secretagogue receptor. Acylation of the Ser3 residue of ghrelin is essential for its biological activities. We hypothesized that acyl-CoA is the fatty acid substrate for ghrelin acylation. Because serum octanoyl-CoA levels are altered by fatty acid oxidation disorders, we examined circulating ghrelin levels in affected patients.

Materials and methods: Blood levels of acyl (A) and des-acyl (D) forms of ghrelin and acylcarnitine of patients with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and glutaric aciduria type II (GA2) were measured.

Results: Plasma acyl ghrelin levels and A/D ratios increased in patients with MCAD deficiency or GA2 when compared with normal subjects. Reverse-phase HPLC confirmed that n-octanoylated ghrelin levels were elevated in these patients.

Conclusion: Changing serum medium-chain acylcarnitine levels may affect circulating acyl ghrelin levels, suggesting that acyl-CoA is the substrate for ghrelin acylation.

Introduction

Ghrelin, an endogenous ligand for the GH secretagogue receptor, is an acylated peptide produced by gastrointestinal endocrine cells (1). Ghrelin is the only peptide known to require a fatty acid modification. Octanoylation of the Ser3 residue is essential for ghrelin-mediated stimulation of GH secretion and regulation of energy homeostasis via increased food intake and adiposity (2, 3). Other than octanoylation (C8:0), the hormone is subject to other types of acyl modification, decanoylation (C10:0), and possibly decenoylation (C10:1) (4, 5). Recently, ghrelin O-acyltransferase (GOAT), which octanoylates ghrelin, was identified (6, 7). The fatty acid substrate that contributes to ghrelin acylation, however, has not been clarified, although the presumed donor is acyl-CoA.

Mitochondrial fatty acid oxidation (FAO) disorders result from genetic defects in transport proteins or enzymes involved in fatty acid β-oxidation (8, 9). The clinical phenotypes have recently been associated with a growing number of disorders, such as Reye syndrome, sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications during pregnancy (10). Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, the most common inherited defect in FAO, causes elevated serum octanoyl-carnitine levels (11), reflecting elevated octanoyl-CoA levels. Glutaric aciduria type II (GA2), which is caused by defects in electron transfer flavoprotein (ETF), ETF-ubiquinone oxidoreductase, or other unknown abnormalities in flavin metabolism or transport, is characterized by elevated serum acylcarnitine levels, including octanoylcarnitine (8, 9). In carnitine palmitoyltransferase II (CPT II) deficiency and very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, serum octanoyl-CoA levels do not increase, but at times actually decrease (8, 9).

We hypothesized that octanoyl-CoA is the fatty acid substrate for ghrelin acylation. To examine this hypothesis, we measured circulating ghrelin levels in patients with MCAD deficiency (MCADD) and GA2.

Materials and methods

Subjects

Five female patients with FAO deficiency (two with MCADD one with GA2, one with CPT II deficiency (12),
and one with VLCAD deficiency) were recruited for this study. The study protocol was approved by the ethics committee on human research at the Kyoto University Graduate School of Medicine. Written informed consent was obtained prior to enrollment.

**Measurement of plasma ghrelin concentrations**

Because FAO patients tend to develop hypoglycemia by fasting, it was difficult to do overnight fasting. Therefore, blood samples for ghrelin analyses were drawn from a forearm vein in the morning after fasting as long as possible. Plasma samples were prepared as described previously (13). Blood samples were immediately transferred to chilled polypropylene tubes containing Na<sub>2</sub>EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Kyoto, Japan: 1000 kallikrein inactivator units/ml = 23.6 nmol/ml (23.6 pM)) and centrifuged at 4 °C. One-tenth volume of 1 M HCl was immediately added to the separated plasma. The acylated and desacylated forms of ghrelin were measured using a fluorescence enzyme immunoassay (FEIA; Tosoh Corp, Tokyo, Japan). The minimal detection limits for acyl and des-acyl ghrelin in this assay system were 2.5 and 10 fmol/ml respectively. The interassay coefficients of variation were 2.9 and 3.1% for acyl and des-acyl ghrelin respectively.

**Reverse-phase HPLC**

Reverse-phase HPLC (RP-HPLC) was performed as described previously (4, 5, 14). Briefly, plasma diluted 50% with 0.9% saline was applied to a Sep-Pak C18 cartridge pre-equilibrated with 0.9% saline. The cartridge was washed with saline and 10% acetonitrile (CH<sub>3</sub>CN solution containing 0.1% trifluoroacetic acid (TFA). Adsorbed peptides were eluted with 60% CH<sub>3</sub>CN mixed and centrifuged, and 5 µl of the supernatant was introduced into liquid chromatography flow of methanol/acetonitrile/water (4:4:2) with 0.05% formic acid using a SII-20AC autoinjector (Shimadzu, Kyoto, Japan). Flow injection and electrospray ionization tandem mass spectrometric (MS/MS) analyses were performed using an API 4000 LC/MS/MS system (AB Sciex, Tokyo, Japan). Positive ion MS/MS analysis was performed in precursor ion scan mode with an m/z value of 85 for the product ion. Data were recorded for 0.7 min after every sample injection and the recorded intensities of the designated ions were averaged using Chemoview Software (Foster City, CA, USA). All samples were measured serially within 1 day.

**Results**

We measured plasma ghrelin concentrations in patients with MCADD and GA2 (Table 1) and also in patients with CPT II and VLCAD deficiency. Elevated C8-acylcarnitine serum levels were observed in MCADD and GA II, whereas they were unchanged or lower in CPT II or VLCAD deficiency (Table 1). Levels of acyl ghrelin but not des-acyl ghrelin appeared to be elevated in patients with MCADD or GA2 in comparison with those in patients with CPT II or VLCAD deficiency, or those in female normal subjects from a previous study.

We then performed RP-HPLC analysis of ghrelin using plasma from patient 1 with MCADD. It demonstrated an eluted peak that corresponded to n-octanoylated human ghrelin-(1–28) in an N-RIA and a C-RIA, indicating that the detected acyl ghrelin was octanoylated (Fig. 1A). When plasma from patient 3 with GA2 was examined using the same method, the N-RIA revealed that the major peak corresponded to n-octanoylated human ghrelin-(1–28) (Fig. 1B). In addition, a small peak, which corresponded to decanoylated ghrelin, was observed in fraction 16 (arrow c), reflecting that serum C10-acylcarnitine levels were also elevated in patient 3 (Table 1).

**Discussion**

Ghrelin is the sole peptide hormone known to have a fatty acid modification. When we started this study in 2007, the catalytic enzyme and fatty acid substrate that mediate ghrelin acylation had not been identified. During this study, the GOAT enzyme was shown to be essential for ghrelin acylation (6, 7). Octanoic acid and octanoyl-CoA were candidates for the fatty acid substrate. We hypothesized that octanoyl-CoA was the substrate, because acylation of ghrelin should be an intracellular process. In fact, Ohgusu et al. (19) showed that acyl-CoA can be the substrate for ghrelin acylation modification using the in vitro assay system. We tested this hypothesis in patients with MCADD and GA2.
which are characterized by higher intracellular octanoyl-CoA levels. Indeed, plasma A/D ratios tended to be elevated in these FAO deficiencies. A relationship between age and ghrelin levels may exist (20, 21). Concerning children, Ikezaki reported that the circulating ghrelin levels tended to correlate negatively with age in children and adolescents, but the correlation was not significant (22). Thus, the relationship has not been confirmed yet. Although we did not compare them directly with those in age- and body mass index (BMI)-matched normal children, they appeared to be higher than those in children with CPT II and VLCAD deficiencies with similar BMIs. BMIs of these patients were comparable to those of normal Japanese female children (23). These findings support the hypothesis that octanoyl-CoA is a primary substrate for ghrelin, although medium-chain triglyceride dietary lipids are a direct source for ghrelin acylation (7, 16, 24). Moreover, GOAT is a membrane-bound molecule in the endoplasmic reticulum (ER). Although how octanoyl-CoA gets into the ER lumen is unclear, Yang et al. (6) speculated that GOAT might mediate the transfer of octanoyl-CoA from the cytosol to the ER lumen. Although serum acylcarnitine levels tended to correlate with acyl ghrelin levels, further studies using more patients with FAO disorders are needed to confirm this relationship.

In addition to \(n\)-octanoylated ghrelin, other molecular forms of the ghrelin peptide exist, including des-acyl ghrelin lacking an acyl modification and such minor acylated ghrelin species as \(n\)-decanoylated ghrelin (Ser3 is modified by \(n\)-decanoic acid) (4, 5). Serum from a patient with GA2 showed the presence of acylated ghrelin that was not octanoylated and was possibly decanoylated (16). In a patient with GA2, intracellular levels of a variety of acyl-CoAs, including octanoyl- and decanoyl-CoAs, were increased, whereas MCADD was associated with specific elevation of octanoyl-CoA levels. In fact, the patient with GA2 had elevated octanoylcarnitine and decanoylcarnitine levels: 1.24 and 1.86 nmol/ml respectively. Nonetheless, the HPLC peak representing \(n\)-decanoylated ghrelin was much smaller than that representing \(n\)-octanoylated ghrelin. Although this is possibly because GOAT acylates ghrelin more efficiently with octanoyl-CoA than decanoyl-CoA, it is more likely because the cross-reactivity between \(n\)-octanoylated and \(n\)-decanoylated ghrelin is 20–25% in the N-RIA. In fact, the HPLC peaks of fraction 15–17 in the C-RIA, which detects similarly both \(n\)-octanoylated and \(n\)-decanoylated ghrelin, were large, strongly suggesting that a substantial amount of \(n\)-decanoylated ghrelin comparable to the elevated decanoylcarnitine level was present. Our observation that acyl ghrelin levels were not elevated in VLCAD and CPT II deficiencies, in which medium-chain acyl-CoAs levels are not higher, supported the idea that GOAT specifically acts on medium-chain acyl-CoAs. Although C16 and C18 levels were not increased in the patient with CPT II deficiency (Table 1), they may be normalized during

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<thead>
<tr>
<th>Subjects</th>
<th>Disease</th>
<th>Age (years)</th>
<th>BMI</th>
<th>Height (cm)</th>
<th>C4 Acylcarnitine (nmol/ml)</th>
<th>C6 Acylcarnitine (nmol/ml)</th>
<th>C8 Acylcarnitine (nmol/ml)</th>
<th>C10:1 Acylcarnitine (nmol/ml)</th>
<th>C10 Acylcarnitine (nmol/ml)</th>
<th>C12 Acylcarnitine (nmol/ml)</th>
<th>C14 Acylcarnitine (nmol/ml)</th>
<th>C16 Acylcarnitine (nmol/ml)</th>
<th>C18 Acylcarnitine (nmol/ml)</th>
</tr>
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<tbody>
<tr>
<td>Patients (n=5)</td>
<td>MCADD</td>
<td>6</td>
<td>15.1</td>
<td>119.5</td>
<td>0.30</td>
<td>0.55</td>
<td>4.61</td>
<td>0.26</td>
<td>2.67</td>
<td>0.02</td>
<td>0.36</td>
<td>1.24</td>
<td>0.03</td>
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<tr>
<td>5</td>
<td>VLCAD</td>
<td>5</td>
<td>14.8</td>
<td>14.8</td>
<td>0.08</td>
<td>0.07</td>
<td>0.10</td>
<td>0.07</td>
<td>0.08</td>
<td>0.08</td>
<td>0.28</td>
<td>0.42</td>
<td>0.21</td>
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<tr>
<td>Normal subjects (n=20); mean ± S.D.</td>
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a stable period in a mild form of CPT II deficiency (25). In fact, this patient did not manifest any marked signs or symptoms at the measurement.

Ghrelin modification with the fatty acid is essential for its biological action. Octanoylation of ghrelin may also be linked to energy homeostasis and fat metabolism. For instance, when serum n-octanoic acid levels increase following fat degradation, ghrelin octanoylation is enhanced, resulting in stimulation of fat synthesis. Thus, ghrelin may play an important role in energy homeostasis through its own fatty acid metabolism. Related to this concept, Kirchner et al. (24) speculated that signaling via GOAT and ghrelin might act as a fat sensor for exogenous nutrients and support fat storage as nutrients are ingested.

FAO deficiency contributes to such clinical problems as sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications (8, 9). Early diagnosis and appropriate management are required to reduce mortality and morbidity associated with this class of disorders. Recently, newborn screening has been expanded in this area. Measuring plasma ghrelin levels may support a diagnosis of MCADD or GA2, for example. Moreover, our results have pathophysiological implications for these disorders. Plasma ghrelin levels are changed by energy demands and food intake (e.g. glucose and fat), and ghrelin affects appetite and adiposity (2, 3). Alterations of plasma ghrelin levels in FAO disorders may reflect and/or influence the patient’s metabolic status. In addition, higher acyl ghrelin levels may affect the Gh/insulin-like growth factor 1 (IGF1) system. There are reports that higher AG levels would increase GH and IGF1 levels (26, 27, 28, 29) and thereby linear growth could be affected. Although none of our patients manifested markedly abnormal growth velocity, we did not measure their serum GH/IGF1 levels. Thus, further studies are warranted to detail a variety of metabolic parameters in this setting.

There are several limitations in this study. At first, the number of FAO patients tested is small. Unfortunately, the incidence of FAO patients in the Japanese population is much smaller than that in Caucasians. Although we asked pediatricians on a nationwide scale, we could successfully collect only five female patients. No adult case has yet been reported in Japan. Secondly, as mentioned above, the normal female subjects were not matched in age or BMI, although patients with MCADD and GA2 exhibited higher plasma A/D ratios than those in child CPT II and VLCAD deficiencies with similar BMIs. To supplement the correlation study, we performed RP-HPLC analysis to prove the increased octanoylation of ghrelin in MCADD and GA2 directly. Further, the presence of n-decanoylated ghrelin is also demonstrated in GA2. Thirdly, the disturbance in the hepatic carbohydrate regulation and the altered peripheral glucose uptake may occur in FAO patients. Hence, abnormal carbohydrate regulation could influence acyl ghrelin levels. Since none of our patients manifested abnormal fasting glucose and HbA1c levels, we speculated that no significant effects occurred.

In summary, we have demonstrated increased levels of acyl ghrelin in patients with MCADD or GA2, which are also characterized by increased intracellular octanoyl-CoA levels. These findings provide mechanistic insights into the biosynthesis of ghrelin. Furthermore, analyzing plasma ghrelin levels may help elucidate pathophysiological processes in FAO deficiencies and aid in the diagnosis of these disorders. Detailed studies using more patients are certainly needed.

**Figure 1** Representative RP-HPLC profiles of ghrelin immuno-reactivity in patients with MCADD (A) and GA2 (B). Closed circles, data obtained using a RIA for a ghrelin C-terminal region (C-RIA); open circles, data obtained using a RIA for a ghrelin N-terminal region (N-RIA). Patient plasma extracts from a Sep-Pak C18 cartridge were fractionated using a Symmetry300 C18 column (5 mm packing, 3.9 × 150 mm, Waters). A linear gradient of 10–60% CH3CN containing 0.1% TFA was passed over the column for 40 min at 1.0 ml/min. The fraction volume was 1.0 ml. Arrows indicate the elution positions of des-acyl human ghrelin-(1–28) (a), n-octanoylated human ghrelin-(1–28) (b), and n-decanoylated ghrelin (c).
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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