Concentration of the n-Octanoylated Active Form of Ghrelin in Fetal and Neonatal Circulation

ICHIRO YOKOTA, SEIKO KITAMURA, HIROSHI HOSODA*, YUMIKO KOTANI AND KENJI KANGAWA*

Department of Pediatrics, School of Medicine, University of Tokushima, Tokushima 770-8503, Japan
*Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka 565-8565, Japan

Abstract. The octanoylation of Ser3 is essential for the biological function of ghrelin. We examined the concentrations of the n-octanoylated active form ghrelin in cord and neonatal blood using an RIA system that specifically recognized n-octanoylated ghrelin, as well as a system that measured the total ghrelin concentration. Plasma levels of active ghrelin in cord blood ranged from 7.7 to 38.4 pmol/l and correlated excellently with those of total ghrelin (r = 0.81, p<0.0001). The active ghrelin/total ghrelin (A/T) ratio ranged from 0.038 to 0.12 (median 0.072). The active ghrelin concentrations negatively correlated with birth body weight (r = –0.34, p = 0.01) and IGF-1 concentrations (r = –0.40, p = 0.003), but did not correlate with growth hormone (GH) concentrations. A considerable level of active ghrelin was detected in premature newborns. Venous cord blood samples showed a significantly higher active ghrelin concentration (p = 0.03) and A/T ratio (p = 0.01) than those in the artery. In neonatal blood, active ghrelin concentrations ranged from 4.6 to 22.6 pmol/l and the A/T ratio ranged from 0.02 to 0.081. These results demonstrate the existence of active-form ghrelin in fetal and neonatal circulation and may suggest the energy supply-dependent regulation of ghrelin expression/secretion in utero.

Key words: Ghrelin, n-Octanoylated form, Cord blood, Neonate

GHRELIN is a 28-amino acid peptide that contains an n-octanoyl modification of Ser3 [1, 2]. This modification is essential for the biological functions of this hormone such as in GH-secretion and appetite stimulation, since the octanoylation of Ser3 is necessary to bind to type 1a growth hormone secretagogue receptor (GHS-R1a) [1, 3]. Measurement of this active-form ghrelin is important for evaluating the regulation and physiological roles of circulating ghrelin under various conditions in humans. However, this octanoylated active-form ghrelin is unstable in blood and easily changes to the des-octanoyl form during sample collection and storage [4]. This makes it difficult to evaluate the mechanism of octanoylation and the significance of its regulation under physiological and pathological states.

We previously reported cord and neonatal blood ghrelin concentrations using a radioimmunoassay (RIA) system that measured total (both octanoyl and des-octanoyl) ghrelin concentrations [5]. Total ghrelin concentrations in cord blood correlated negatively with anthropometric and hormonal parameters that represent the nutritional status of fetuses, which suggested that fetal ghrelin concentrations were regulated in a fetomaternal energy transport-dependent manner. However, the presence of n-octanoylated active-form ghrelin during the perinatal period and its relation to des-octanoylated ghrelin has not been well examined. Thus, measurement of active-form ghrelin concentrations in the fetal and neonatal circulation is very important for better understanding the role of ghrelin during this period [6].

In the present study, we measured n-octanoylated active-form ghrelin concentrations in cord and neonatal blood using an octanoylated form-specific RIA system we have developed [2] and examined its relation to total ghrelin and other hormonal and anthropometric parameters in fetuses and neonates.
Subjects and Methods

Subjects

Venous cord blood samples were obtained from 54 full-term newborns (34 males and 20 females, gestational age 37.0–41.6 weeks, birth weight 2,206–4,326 g, birth length 44.0–54.5 cm). They were the same subjects as those in whom we had previously reported the results of total ghrelin concentrations [5]. Forty-four of the newborns were classified as appropriate for gestational age (AGA), 7 were small for gestational age (SGA) and 3 were large for gestational age (LGA) according to the classification described previously [5]. In addition, 5 premature newborns (3 males and 2 females, gestational age 30.6–35.3 weeks, birth weight 778–2,154 g, birth length 35.0–47.5 cm, 2 SGA and 3 AGA) were included in this study. In another 8 full-term newborns, both arterial and venous cord blood samples were collected. Blood samples for ghrelin assay were collected in chilled tubes containing EDTA·2Na (1 mg/ml) and aprotinin (500 U/ml). Plasma was separated at 4°C immediately, acidified with 1N HCl (1:10 relative to the plasma volume) to stabilize octanoyl modification, and stored at –80°C until analysis. Every procedure was performed within 30 minutes after birth for each sample collection. Serum was simultaneously separated for other hormone assays. Neonatal samples were obtained from 47 full-term healthy neonates (27 males and 20 females, postnatal day 3–8) as described previously [5]. Among them, the difference in the ghrelin concentration between cord and neonatal blood was compared in 27 neonates. These samples were also immediately separated at 4°C and acidified before freezing. The study protocol was approved by the ethical committee of The University of Tokushima, School of Medicine, and all of the parents of the newborns gave their written informed consent before enrollment.

Ghrelin and other hormone assays

We measured active and total ghrelin levels using two RIA systems [2, 5]. Serum GH and insulin-like growth factor-I (IGF-I) were determined using immunoradiometric assay kits (Daichi Radioisotope Laboratories, Tokyo, Japan). Serum insulin-like growth factor-II (IGF-II) was determined using an enzyme-linked immunosorbent assay kit (Diagnostic Systems Laboratories, Inc., Sinsheim, Germany). Serum immunoreactive insulin (IRI) was determined using an immunoradiometric assay kit (Eiken Chemical, Tokyo, Japan). Serum insulin-like growth factor binding protein-3 (IGFBP-3) was determined using an RIA kit (Cosmic Corp., Tokyo, Japan). Serum leptin was determined using an RIA kit (Linco Research, Inc., St. Charles, MO).

Statistical analysis

All quantitative data are presented as the median and range. Pearson’s correlations were used to examine relationships among clinical growth-related parameters and hormone levels. Differences between groups were evaluated by the Mann-Whitney U-test or Wilcoxon’s signed rank test. Significance was considered to be p<0.05. The analysis was conducted with StatView software (version 5.0 for Windows, SAS Institute Inc., Cary, NC) or SPSS software (version 11.0J for Windows, SPSS Inc., Chicago, IN).

Results

In 54 cord blood samples from full-term neonates, the plasma concentrations of active ghrelin ranged from 7.7 to 38.4 pmol/l (median 14.5 pmol/l). The A/T ratios ranged from 0.038 to 0.12 (median 0.072). Active ghrelin concentrations showed excellent correlations with total ghrelin concentrations (r = 0.81, p<0.0001) and the A/T ratio (r = 0.55, p<0.0001) (Fig. 1A). Active ghrelin concentrations in SGA newborns were significantly higher than those in AGA and LGA newborns (p = 0.02 and p = 0.02, respectively). No significant correlation was observed between active ghrelin and GH concentrations (r = 0.24, p = 0.10).

In 5 premature neonates, total ghrelin concentrations ranged from 183.9 to 311.9 pmol/l (median 14.5 pmol/l). The A/T ratios ranged from 0.038 to 0.12 (median 0.072). Active ghrelin concentrations showed excellent correlations with total ghrelin concentrations (r = 0.81, p<0.0001) and the A/T ratio (r = 0.55, p<0.0001) (Fig. 1A). Active ghrelin concentrations were inversely correlated with birth weight (r = 0.34, p = 0.01), placental weight (r = 0.31, p = 0.03), birth weight (BW)/birth length (BL) ratio (r = 0.34, p = 0.01), Kaup index (r = 0.29, p = 0.04) and IGF-I concentration (r = 0.40, p = 0.003) (Table 1). Active ghrelin concentrations in SGA newborns were significantly higher than those in AGA and LGA newborns (p = 0.02 and p = 0.02, respectively). No significant correlation was observed between active ghrelin and GH concentrations (r = 0.24, p = 0.10).
considerable levels of active and total ghrelin were detected in premature newborns.

In 47 AGA newborns (44 full-term and 3 premature), total ghrelin concentrations did not significantly correlate with gestational age ($r = 0.06, p = 0.67$). However, active ghrelin concentrations and the A/T ratio significantly correlated with gestational age ($r = 0.35, 0.44$ and $p = 0.02, 0.002$, respectively). These correlations were evident even when we only considered the 44 full-term newborns ($r = 0.22, 0.43, 0.39$ and $p = 0.16, 0.003, 0.009$ for total ghrelin levels, active ghrelin levels, and A/T ratio, respectively) (Fig. 2).

The differences in active ghrelin concentrations and the A/T ratio between arterial and venous cord blood were examined in 8 full-term newborns. The plasma levels of active ghrelin in the vein (median 21.7 pmol/l, range 15.4 to 32.2 pmol/l) were significantly higher than those in the artery (median 17.8 pmol/l, range 8.8 to 34.3 pmol/l, $p = 0.03$). Simultaneously, the A/T ratio in the vein was significantly higher than that in the artery ($p = 0.01$) (Fig. 3).

In 47 neonates, the plasma levels of active ghrelin ranged from 4.6 to 22.6 pmol/l (median 11.6 pmol/l) and the A/T ratio ranged from 0.02 to 0.081 (median 0.048). Active ghrelin concentrations correlated with total ghrelin concentrations ($r = 0.61, p<0.001$) (Fig. 1B) and the A/T ratio ($r = 0.57, p<0.001$). A comparison of the ghrelin concentrations in venous cord blood and neonatal blood in 27 newborns showed that, while there was no significant difference in active ghrelin concentrations, the A/T ratio in neonatal blood was significantly lower than that in cord blood ($p<0.001$). The active ghrelin concentrations in neonates did not significantly correlate with percent body weight loss from birth to the sampling day ($r = 0.06, p = 0.67$), calorie intake per body weight on the sampling day ($r = 0.11, p = 0.48$), or the mean daily body weight gain during the first month of life ($r = 0.19, p = 0.19$).

**Discussion**

Stabilization of octanoylated Ser3 during sample collection is essential for measuring the active form of ghrelin. Acidification is a simple, reliable procedure for protecting the acylated form of ghrelin from deacylation [4]. Sample collection without prompt plasma separation and acidification might be inappropriate for measurement of the active-form ghrelin concentration [9].
In our study, we found an excellent correlation between the active and total ghrelin concentrations. This suggests that our previous observations using total ghrelin concentrations as a parameter of ghrelin bioactivity adequately reflect the regulation of it during the perinatal period. In fact, correlation studies between active ghrelin concentrations and anthropometric or other hormonal parameters have shown results similar to those observed for total ghrelin concentrations [5]. However, the results of ghrelin knockout mouse analyses did not show significant changes of growth [10, 11]. Thus, further investigation is still needed to determine whether this regulation of circulating ghrelin bioactivity has obvious physiological effects for the regulation of fetal growth. The relatively weak correlation between active ghrelin concentrations and these parameters in some cases may result from the scattering of data due to the instability of active ghrelin in sample collection, even with our prompt and careful procedure. Alternatively, the total ghrelin concentration, rather than the active ghrelin concentration, might be appropriate for evaluating the relationship between the expression/secretion of ghrelin and the mid- or long-term effects of nutritional conditions that are represented by these anthropometric or hormonal parameters.

Although we examined only a few samples, considerable levels of active ghrelin were detected in those
from premature newborns. This suggests that the mechanism for the n-octanoylation of Ser3 is present even during the premature stage. In addition, we found a positive correlation between gestational age and both the active ghrelin concentration and the A/T ratio. In contrast, a positive correlation was not seen between gestational age and total ghrelin concentrations. This may suggest either the maturation of n-octanoylation system during gestation or relatively earlier degradation of active form ghrelin during earlier gestational stage.

In our previous study, we found significantly higher total ghrelin concentrations in venous cord blood than in the artery, and suggested that the placenta may play some roles as one of the sources of ghrelin in the fetal circulation, in addition to other fetal organs such as the lung and pancreas [5, 12–14]. In this study, we observed a higher active ghrelin concentration and A/T ratio in the vein. These findings suggest that ghrelin may be produced in the placenta with a relatively higher A/T ratio and a small part of it is transferred to the fetal circulation, like leptin [15]. The extent of the contribution of the placenta to the cord blood ghrelin concentration will need to be studied further. Another interesting possibility is that some circulating des-octanoylated ghrelin might be octanoylated through the placenta. Furthermore, a higher A/T ratio in venous cord blood than in the corresponding neonatal blood might be explained in part by a difference in the sampling location. For example, the A/T ratio in plasma is much lower than that in the stomach in the rat [2].

In summary, we found significant levels of n-octanoylated ghrelin in fetal and neonatal blood. An excellent correlation between the active and total ghrelin concentrations suggested that measurement of the total ghrelin concentration is adequate for analyzing the regulation of ghrelin bioactivity during the perinatal period. The maturation of the n-octanoylation system during the development of each organ needs to be studied further to better understand the detailed actions of ghrelin during this period.

Acknowledgments

We thank Dr. K. Maeda and Professor M. Irahara (Department of Obstetrics & Gynecology, School of Medicine, University of Tokushima) for their support with the sample collection. This work was supported in part by a grant from the Foundation for Growth Science (Tokyo, Japan).

References


