

Acta Medica Okayama

Volume 64, Issue 2

2010

Article 7

APRIL 2010

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Abstract

Ciliary neurotrophic factor (CNTF) has been shown to decrease food intake in mouse models of obesity and to improve insulin sensitivity. It is well known that tight regulation of glucose metabolism is essential for successful gestational outcomes (e.g. fetal growth), and that abnormal insulin resistance is associated with preeclampsia (PE). To investigate the possibility that CNTF might be involved in the regulation of insulin resistance during pregnancy, circulating levels of CNTF were assessed in non-pregnant, normal pregnant, postpartum, and pregnant women with PE. Sera from healthy non-pregnant women (n10), pregnant women (n30:1st trimester;n10, 2nd trimester n10;3rd trimester;n10), postpartum women (n10), and patients with PE (n11) were studied with Western blotting. Circulating CNTF was detected by Western blotting, and the levels of CNTF in pregnant women were decreased as compared with those in non-pregnant women, and tended to decrease as pregnancy progressed. A significant decrease was found in PE as compared with normal pregnancy. Circulating CNTF might be associated with physiological and abnormal insulin resistance during pregnancy.

KEYWORDS: ciliary neurotrophic factor, insulin sensitivity, pregnancy, preeclampsia, placenta

Original Article

Circulating Levels of Ciliary Neurotrophic Factor in Normal Pregnancy and Preeclampsia

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Ciliary neurotrophic factor (CNTF) has been shown to decrease food intake in mouse models of obesity and to improve insulin sensitivity. It is well known that tight regulation of glucose metabolism is essential for successful gestational outcomes (*e.g.* fetal growth), and that abnormal insulin resistance is associated with preeclampsia (PE). To investigate the possibility that CNTF might be involved in the regulation of insulin resistance during pregnancy, circulating levels of CNTF were assessed in non-pregnant, normal pregnant, postpartum, and pregnant women with PE. Sera from healthy non-pregnant women ($n = 10$), pregnant women ($n = 30$: 1st trimester; $n = 10$, 2nd trimester $n = 10$; 3rd trimester; $n = 10$), postpartum women ($n = 10$), and patients with PE ($n = 11$) were studied with Western blotting. Circulating CNTF was detected by Western blotting, and the levels of CNTF in pregnant women were decreased as compared with those in non-pregnant women, and tended to decrease as pregnancy progressed. A significant decrease was found in PE as compared with normal pregnancy. Circulating CNTF might be associated with physiological and abnormal insulin resistance during pregnancy.

Key words: ciliary neurotrophic factor, insulin sensitivity, pregnancy, preeclampsia, placenta

Ciliary neurotrophic factor (CNTF) is a 22-kDa cytokine belonging to the interleukin (IL)-6 family [1, 2], and is mainly expressed in glial cells within the central and peripheral nervous systems [3, 4]. Barbin *et al.* (1984) have described the neurotrophic activity of CNTF purified from chick eye [5], and ablation of CNTF gene by homologous recombination in mice (Masu *et al.* (1993)) has shown that progressive atrophy and loss of motor neurons occurs in homozygotes [6]. In addition to functions in

the central nervous system, there have been several reports regarding the detection of circulating CNTF in healthy individuals, patients with septic shock, systemic lupus erythematosus, rheumatoid arthritis, multiple myeloma, and amyotrophic lateral sclerosis [7–11]; however, the physiological relevance of circulating CNTF still needs to be established. Recent studies have revealed that CNTF is able to bind to a specific receptor that is widely distributed throughout the body and that consists of CNTF-specific components [1]: gp130 and leukemia inhibitory factor (LIFR) [1, 12, 13]. No report regarding circulating levels of CNTF in pregnancy has been published. Importantly, exogenously administered CNTF has

Received October 30, 2009; accepted November 30, 2009.

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been found to decrease food intake in mouse models of obesity and to improve peripheral insulin resistance by decreasing hepatic steatosis [14–19].

During pregnancy, tight regulation of glucose metabolism is a prerequisite for appropriate fetal growth and for maternal well-being. Insulin resistance in normal pregnancy, which is considered to be “physiological,” peaks at the third trimester and rapidly returns to pre-pregnancy levels after parturition. PE, which occurs in approximately 5 to 10% of all pregnant woman, is one of the most common pregnancy-associated disorders [20], and several lines of evidence suggest that insulin resistance is associated with PE [20, 21]. Our data indicate that circulating CNTF is decreased during pregnancy when physiological insulin resistance is present, and the levels of CNTF in PE were significantly decreased as compared with matched normal pregnancy.

Materials and Methods

Human subjects. Retrospectively and randomly selected sera from 10 healthy women, 30 healthy pregnant women without PE, 10 postpartum women, and ten PE cases were analyzed for circulating CNTF levels. PE was diagnosed and classified according to the technical bulletin of the American College of Obstetricians and Gynecologists and the National High Blood Pressure Education Program Working Group Report on High Blood Pressure in Pregnancy [22, 23]. Sera were separated immediately after the blood collection by centrifugation, and were stored frozen at -20°C until use. **Normal pregnant samples** were collected from pregnant women at various gestational weeks at their regular visits, and non-pregnant samples were obtained from volunteer women. Postpartum samples were collected at 4 days after delivery. In all but one PE case, blood was collected just prior to delivery. From one particular PE case, sera were collected at 2 different times (at hospitalization and pre-operation (emergency cesarean section)). At hospitalization, the blood pressure of the patient was 160/100 mmHg. When we decided to perform a cesarean section due to non-reassuring fetal status, the blood pressure of the patient was 200/110 mmHg. All pregnant samples were collected before rupture of the membrane and the initiation of labor to avoid possible influence.

This study was approved by the Institutional Ethical Review Board of Okayama University Hospital (project # 186, June 21th, 2004), and all subjects provided informed consent.

Western blotting. Western blotting of serum samples was performed as follows. Sera were diluted at a 1:20 ratio with Phosphate-Buffered Saline (PBS), mixed with 5x SDS-PAGE loading buffer, boiled for 5 min, and separated by 10–20% SDS gradient polyacrylamide gels. After the separation, proteins were electroblotted onto a nitrocellulose membrane (Hybond-C. GE healthcare UK Ltd., Buckinghamshire, UK), even-loading was confirmed by Ponceau-S reversible staining, and non-specific binding was blocked at room temperature for 2h with 5% BSA. Thereafter, membranes were incubated at 4°C overnight with chicken anti-human CNTF polyclonal antibody (1:2000, Abcam Plc., Cambridge, UK) in the blocking buffer. The membranes were then washed and incubated for 2h at room temperature with 1:10000 diluted horse radish peroxidase-conjugated donkey anti-chicken IgY (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), washed, and specific signals were visualized with Super signal[®] West pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA). The signal intensities of the specific bands were densitometrically analyzed with Scion image computer software (Scion Corp., Frederick, MD, USA). The reference serum was prepared from a healthy non-pregnant woman and was loaded onto every blot; the signal intensity of each sample was compared with this reference, then expressed as an arbitrary unit; sample/standard ratio. As a positive control, recombinant human CNTF (Pepro Tech Inc., Rocky Hill, NJ, USA) was used.

Statistical analysis. Statistical significance was analyzed using Stat Mate III (Atms Co., Ltd., Tokyo, Japan). For the analysis of 2 independent groups (e.g. normal pregnant v.s. PE), Mann-Whitney’s U test and chi-square test were used. For the analysis of CNTF levels at various gestational ages, the Kruskal-Wallis test was used. *P*-values less than 0.05 are considered to be significant.

Results

Characteristics of patients with PE and con-

trol (normal pregnancy). Patient characteristics were compared between PE and healthy pregnant women (Table 1). No significant differences were found for the average maternal age, the percentage of primigravida, height, the percentage of smokers. The blood pressure of PE at the time of blood sampling ($176 \pm 4.92 / 107 \pm 3.78$) was significantly higher than that of normal pregnancy ($104 \pm 2.86 / 46.2 \pm 2.45$). Body weight and BMI before pregnancy, body weight at the time of blood sampling for CNTF levels (gestational weeks: normal pregnancy; 31.9 ± 0.70 (median 32, range 29–36), PE; 31.7 ± 0.90 (median 32, range 28–36), not significant, and body weight at delivery of PE were significantly heavier than that of normal pregnancy. However, body weight gain at the time of blood sampling and body weight gain at delivery of PE were significantly smaller than that for normal pregnancy. Gestational age at delivery and the birth weight of the newborns of PE (32.8 ± 0.84 weeks, $1,457 \pm 133$ grams) were significantly smaller than those of normal pregnancy (38.7 ± 0.52 weeks, $3,240 \pm 110$ grams).

Detection of circulating CNTF in non-pregnant and pregnant women. We first attempted to detect circulating CNTF by Western blotting using specific antibody in healthy non-pregnant women. A

discrete band at the expected molecular weight of 22.7 kDa was detected (Fig. 1A). In addition, several nonspecific bands were present. Next, in order to assess the influence of pregnancy on circulating CNTF levels, sera from pregnant women were compared with non-pregnant controls. CNTF was detected in both statuses. Circulating levels of CNTF in pregnant women (gestational weeks: median 25, range 10–39) were significantly decreased as compared with non-pregnant women (0.78 ± 0.08 v.s. 0.56 ± 0.04 , mean \pm S. E. M.) (Fig. 1B, C). To observe the effect of gestational weeks, circulating levels of CNTF were evaluated in each trimester. The levels of circulating CNTF were significantly decreased in the 2nd and 3rd trimesters as compared with the 1st trimester (1st trimester; 0.89 ± 0.09 , 2nd trimester; 0.66 ± 0.06 , 3rd trimester 0.56 ± 0.09 , mean \pm S. E. M.) (Fig. 2A, B). No significant difference was seen between non-pregnant women and the 1st trimester (data not shown). We also looked at the postpartum CNTF levels. Circulating levels of CNTF remained low at 4 days after delivery (Fig. 2A, B).

Decreased circulating levels of CNTF in PE. We next assessed the levels of circulating CNTF in PE. PE samples were compared with gestational age-matched normal pregnant samples.

Table 1 Characteristics of PE patients and healthy pregnant women

	Normal Preg. (n = 9)	PE (n = 10)	P value
Age (years old)	31.8 ± 0.73 (29, 20–40)	33.9 ± 1.48 (34, 25–41)	NS
Primigravida number (%)	3 (33)	5 (45)	NS
Systolic blood pressure (mmHg)	104 ± 2.86 (102, 94–120)	176 ± 4.92 (181, 160–200)	< 0.01
Diastolic blood pressure (mmHg)	46.2 ± 2.45 (50, 36–58)	107 ± 3.78 (109, 96–142)	< 0.01
Height (cm)	162 ± 2.27 (160, 150–170)	158 ± 1.87 (160, 150–167)	NS
Body weight (prepregnancy) (kg)	56.6 ± 2.91 (56, 47–76)	65.0 ± 5.21 (60, 44–95)	< 0.01
Body weight* (kg)	65.9 ± 2.79 (65, 52–83)	71.2 ± 4.33 (65, 55–99)	< 0.01
Body weight at delivery (kg)	66.1 ± 2.70 (66, 55–83)	71.3 ± 4.35 (65, 55–99)	< 0.01
Body weight gain* (kg)	9.25 ± 1.11 (8.9, 5.4–14.8)	6.22 ± 1.70 (7.6, –4.5–14)	< 0.01
Body weight gain at delivery (kg)	9.44 ± 1.97 (11, 6–16)	6.24 ± 1.83 (7.6, –4.5–14)	< 0.01
Smoking (%)	1 (11)	1 (9.1)	NS
BMI (prepregnancy)	21.4 ± 0.84 (21.4, 18.3–26.2)	25.8 ± 1.84 (25.1, 19.6–37.1)	< 0.01
Gestational age* (weeks)	31.9 ± 0.70 (32, 29–36)	31.7 ± 0.90 (32, 28–36)	NS
Gestational age at delivery (weeks)	38.7 ± 0.52 (38, 37–41)	32.8 ± 0.84 (32, 28–36)	< 0.01
Birth weight (gram)	$3,240 \pm 110$ (3,140, 2,936–4,048)	$1,457 \pm 133$ (1,288, 749–2,120)	< 0.01

Unless noted, values are the mean \pm S. E. M (median, range). Normal Preg. ; gestational-age-matched healthy pregnant women. PE, preeclampsia; BMI, body mass index; NS, not significant. * at the time of blood sampling for CNTF levels [Gestational weeks for normal pregnancy: 31.9 ± 0.70 (median 32, range 29–36), for PE: 31.7 ± 0.90 (32, 28–36)] Statistical analysis was conducted by Mann-Whitney’s U test except for “primigravidanumber” and “smoking” which were conducted by chi-square test.

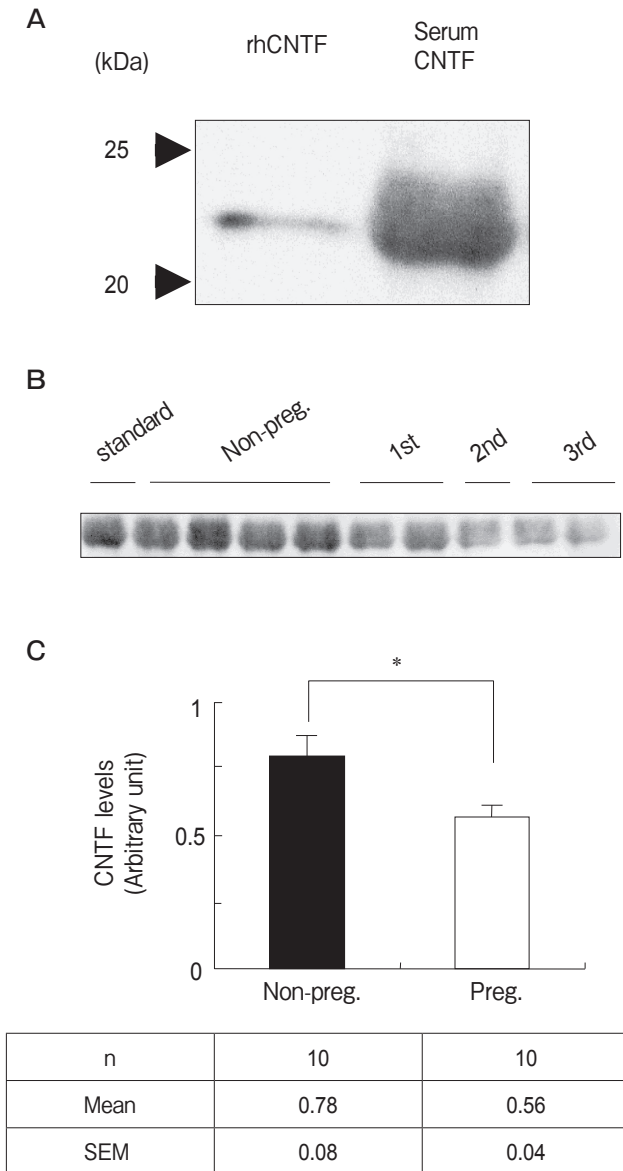


Fig. 1 Detection of circulating human CNTF by Western blotting & circulating levels of CNTF were decreased during pregnancy. **A**, CNTF protein was detected by Western blotting in human serum (non-pregnant healthy individual). A recombinant human CNTF (rhCNTF) was used as a positive control; **B**, Circulating levels of CNTF was assessed by Western blotting. Sera from various gestational stages were compared with non-pregnant samples. Signal intensities of non-pregnant group were higher than that of pregnant group. Standard, healthy 24 years old woman; Non-preg., non-pregnant healthy women; 1st, 1st trimester; 2nd, 2nd trimester; 3rd, 3rd trimester; **C**, Circulating levels of CNTF in pregnant women were significantly decreased as compared with non-pregnant women. Densitometric quantification of the blots is shown. Non-preg., non-pregnant healthy women; Preg, pregnant healthy women from various gestational stages. * $p < 0.05$.

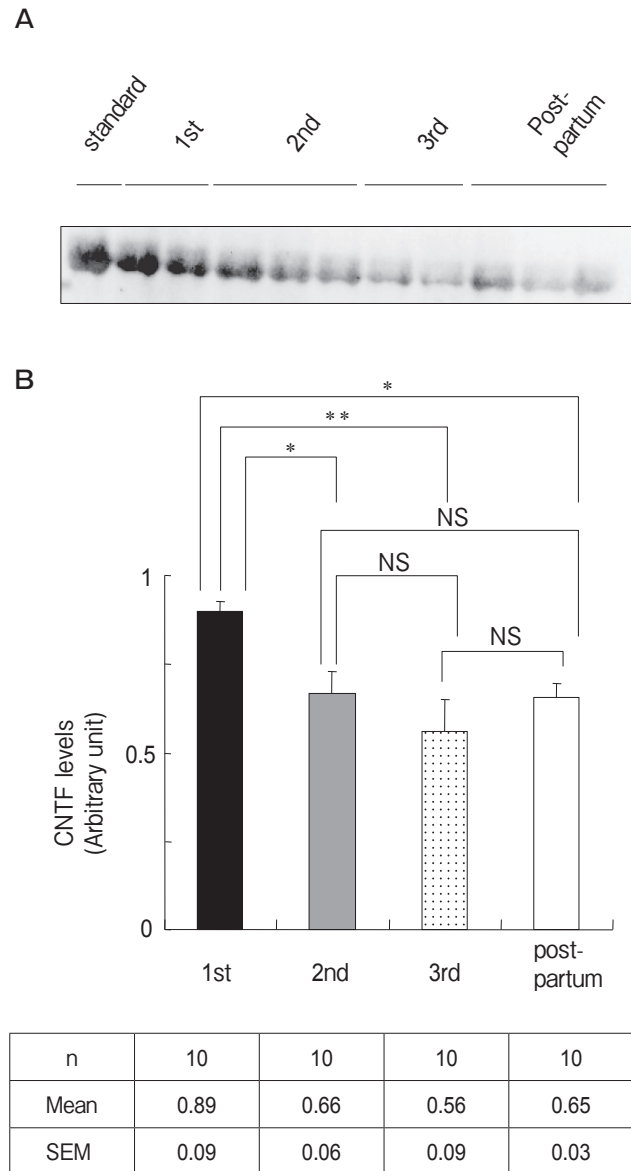


Fig. 2 Circulating levels of CNTF was decreased as pregnancy progressed. **A**, Circulating levels of CNTF was assessed by Western blotting, and grouped into each trimester. CNTF levels appeared to be decreased toward later gestation. Circulating levels of CNTF were remained at low levels 4days after delivery. Standard, healthy 24 years old woman; 1st, 1st trimester; 2nd, 2nd trimester; 3rd, 3rd trimester; **B**, Significant decrease of levels of CNTF between 1st and 2nd, or 1st and 3rd trimester, or 1st and postpartum. Densitometric quantification is shown. * $p < 0.05$, ** $p < 0.01$. NS, not significant.

Circulating levels of CNTF in PE cases were significantly lower than those in normal pregnant women (0.34 ± 0.05 v.s. 0.18 ± 0.01 , mean \pm S. E. M, $p <$

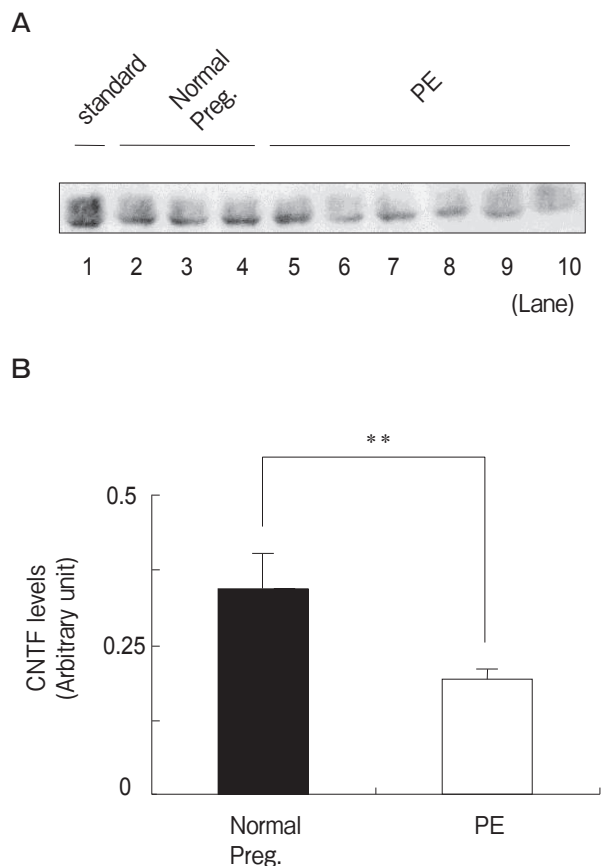
0.01) (Fig. 3A B). In addition, the samples that were loaded on lanes 5 and 9 were collected from the same patient at hospitalization (gestational week 26) and just prior to the emergency cesarean section (gestational week 28), (Fig. 3A). The level of CNTF in lane 9 was obviously lower than that of lane 5, indicating that CNTF levels might be inversely correlated with the severity of the condition (repeated by loading side by sides).

Correlation of circulating CNTF levels and body weight gain at the 3rd trimester. Obesity is known to be associated with insulin resistance and gestational hypertension; therefore, the relation between circulating CNTF and body weight was studied. No significant correlation was found between circulating CNTF levels and BMI in non-pregnant women ($r = 0.15$, not significant) (Fig 4A). Correlations between CNTF levels and body weight, CNTF levels, and blood glucose levels also were not significant (data no shown). The body weight gain of normal pregnancy (non-pregnant to 3rd trimester), however, was significantly correlated with circulating levels of CNTF ($r = 0.87$, $p < 0.01$) (Fig. 4B). There was no significant correlation between CNTF levels and the body weight gain of PE cases ($r = 0.24$, not significant), (Fig. 4C).

Discussion

Recently, several new key regulators of insulin sensitivity have been described, including adipokines (e.g. TNF α , IL-6, leptin, adiponectin, and resistin) [24, 25]. These adipokines regulate insulin resistance [24, 25], and it has been of great interest how these adipokines are associated with the pathogenesis of PE [26–31] and gestational diabetes mellitus [32, 33] in relation to the regulation of insulin resistance during pregnancy.

CNTF is one of the newest potential humoral factors that regulate insulin sensitivity [14–19]. Recombinant CNTF (CNTFAX15) function via the central and peripheral pathway results in anti-obesogenic effects and an improvement of insulin sensitivity [16, 19, 34–37]; CNTF is therefore believed to have 2 sites of action: the central nervous system and peripheral organs. CNTF receptors localized on hypothalamic neurons are involved in feeding and weight control. CNTF functions centrally via gp130



n	9	11
Mean	0.34	0.18
SEM	0.05	0.01

Fig. 3 Decreased circulating levels of CNTF in preeclampsia. **A**, Circulating levels of CNTF was assessed by Western blotting. Circulating levels of CNTF in PE cases appeared to be decreased as compared with gestational-age-matched normal pregnant women. Standard, healthy 24 years old woman; Normal Preg, gestational-age-matched normal pregnant women; PE, preeclampsia; **B**, Circulating levels of CNTF in PE cases were significantly lower than healthy normal pregnant women. Densitometric quantification is shown. Lane 5 (at hospitalization) and Lane 9 (at pre-operation for emergency cesarean section) were collected from the same patient. Note that the intensity of lane 9 was lower than that of lane 5. ****** $p < 0.01$.

receptor signaling in proopiomelanocortin (POMC)-expressing neurons in the hypothalamus to reduce AMP-activated kinase (AMPK) activity [38]. In addition, CNTF decreases feeding and weight by suppressing neuropeptide Y (NPY) [34] and inducing

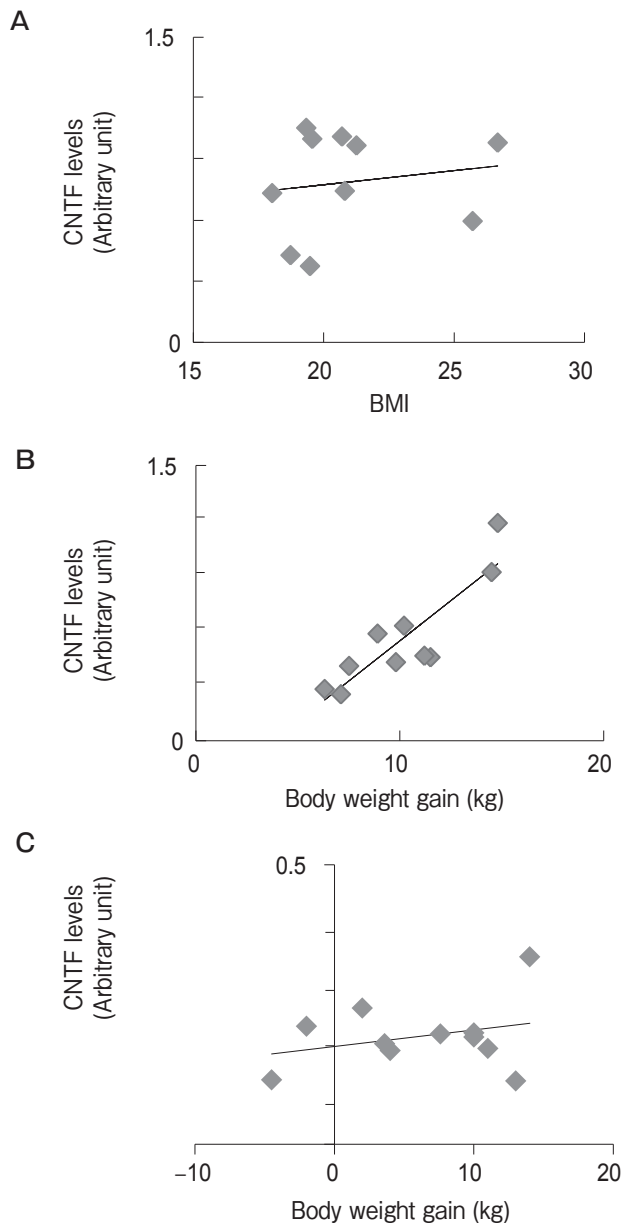


Fig. 4 Correlation of circulating CNTF levels and BMI or body weight gain. **A**, Correlation of circulating CNTF levels and BMI in non-pregnant women is shown. No significant correlation was found between circulating CNTF levels and BMI in non-pregnant women ($r = 0.15$, not significant). BMI, body mass index; **B**, Correlation of circulating CNTF levels and body weight gain at 3rd trimester is shown. Body weight gain (non-pregnant to 3rd trimester) was positively correlated with circulating levels of CNTF at 3rd trimester ($r = 0.87$, $p < 0.01$). Body weight gain, body weight before pregnancy-body weight at 3rd trimester; **C**, Correlation of circulating CNTF levels and body weight gain at 3rd trimester is shown in PE cases. No significant correlation was found between circulating CNTF levels and body weight gain in PE cases ($r = 0.24$, not significant).

neurogenesis in the arcuate nuclei [16]. In peripheral organs such as skeletal muscle [35] and adipocytes [36], CNTF elevates AMPK activity, which results in increased fatty acid oxidation. Furthermore, CNTF acts to decrease steatosis of the liver [19] and lipid build-up in skeletal muscle [35], which results in improved insulin sensitivity. It is therefore likely that CNTF is involved in the regulation of insulin resistance by both central and peripheral actions; accordingly, it is also very likely that CNTF is involved in the regulation of insulin resistance during pregnancy. These findings led us to assess the circulating levels of CNTF during pregnancy.

Circulating levels of CNTF were decreased during pregnancy, which is consistent with the idea that CNTF acts to improve insulin resistance, as “physiological insulin resistance” occurs during pregnancy. Also, during pregnancy, “hemodilution” could affect the concentrations of circulating factors. While it is possible that decreased CNTF levels in normal pregnancy are a result of “hemodilution,” lowered circulating levels of CNTF could cause attenuated peripheral actions of CNTF and thus might act to decrease insulin sensitivity in pregnant women. Although the evidence is insufficient, the possibility remains that synthesis or secretion of circulating CNTF might be altered during pregnancy.

We also found that circulating levels of CNTF in PE were significantly lower than those in normal pregnancy. It has been well accepted that “hemoconcentration [37]” occurs in PE that should lead to increased concentrations of circulating factors. Decreased circulating levels of CNTF in PE, therefore, might be indicative of decreased production/secretion of CNTF. Interestingly, we were able to assess sera from one PE case at 2 stages. Circulating levels of CNTF lowered as the severity of PE increased, supporting this idea. To prove that the production or secretion of circulating CNTF is regulated in some way during pregnancy, we must clarify the source of circulating CNTF, which remains unknown.

It is well known that excess body weight gain is often associated with insulin resistance and also with gestational hypertension [39]. Therefore, the correlation between circulating CNTF levels and body weight gain was studied. In non-pregnant women, there was no significant correlation between BMI and

CNTF levels were found. Interestingly, a significant positive correlation was found between body weight gain during pregnancy (with no complication) and the levels of CNTF at the time of blood sampling. In PE, however, no significant correlation between body weight gain and CNTF levels at the time of delivery was found (Fig. 4C). Hypothesizing that there are mechanisms regulating insulin resistance during pregnancy, factors that improve or deteriorate insulin resistance might be assumed. Under this assumption, such mechanisms might be affected in PE, and CNTF could be a factor that improves insulin resistance during normal pregnancy. On the other hand, body weight gain in PE is smaller than that in normal pregnancy (6.24 ± 1.83 vs 9.25 ± 1.11 , $p < 0.01$, Table 1). It is possible that patients at high risk for PE are usually under strict nutritional control (*e.g.* calorie and salt intake), and thus body weight gain is restricted. This phenomenon might be the cause of the decreased CNTF levels in the PE group; however, comparing similar body weight gain cases between the 2 groups, the levels of CNTF in PE were significantly lower than those in normal pregnancy (CNTF 0.37 ± 0.08 PE 0.19 ± 0.03 , $n = 6$, $p < 0.05$, (range 7–16 kg, body weight gain of control vs PE: 12.2 ± 1.3 vs 10.1 ± 0.9 , not significant)). Thus, CNTF levels might be closely associated with body weight gain during pregnancy, the underlying mechanism of which might be different from non-pregnant status. To clarify this point, however, it will be necessary to increase the number of samples in future studies. In addition, HOMA-IR was compared between PE and the control group, and it was found to be not significantly different (data not shown).

It is well known that the placenta secretes a number of important peptide hormones (*e.g.* hPL, hCG *etc.*), and is easily accessed after delivery; it is therefore tempting to examine the expression of CNTF in the placenta. Our preliminary studies have shown the expression of CNTF mRNA and protein by RT-PCR and Western blotting (data not shown). In addition, immunohistochemical study of normal and PE placentas indicated that immunoreactive CNTF is decreased in PE placentas (data not shown). Clearly further studies are required, but the placenta is interesting as a possible source of CNTF during pregnancy.

Taken together, we believe that our study provides new and important information regarding the func-

tional significance of circulating CNTF in the regulation of insulin resistance during pregnancy, which might shed new light on the extra-neural functions of CNTF.

Acknowledgments. This work was supported in part by the Japan Ogyaa Donation Foundation.

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