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Plasma Levels of Biotin Metabolites Are Elevated in Hemodialysis Patients with Cramps

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Patients with renal failure undergoing hemodialysis (HD) are susceptible to muscle cramps during and after HD. Muscle cramps are defined as the sudden onset of a prolonged involuntary muscle contraction accompanied by severe pain. Through HD, water-soluble vitamins are drawn out with water. Since biotin, a water-soluble vitamin, plays an essential role as one of the coenzymes in producing energy, we have hypothesized that deficiency of biotin may be responsible for HD-associated cramps. We previously reported that biotin administration ameliorated the muscle cramps, despite the elevated plasma biotin levels before HD and biotin administration, as judged by an enzyme-linked immunosorbent assay (ELISA). However, the ELISA measures not only biotin but also total avidin-binding substances (TABS) including biotin metabolites. In the present study, we determined biotin in HD patients as well as healthy controls, using a newly developed method with ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The plasma samples were collected from 28 HD patients (16 patients with cramps and 12 patients without cramps) before HD and biotin administration and from 11 controls. The results showed that the accumulation of biotin and TABS in plasma of HD patients compared to controls. Importantly, the levels of biotin metabolites, *i.e.* TABS subtracted by biotin, increased significantly in patients with cramps over those without cramps. Moreover, the levels of biotin metabolites were significantly higher in patients with a poor response to administered biotin, compared to those with a good response. We propose that accumulated biotin metabolites impair biotin's functions as a coenzyme.

Keywords: cramp; ELISA; hemodialysis; plasma biotin; UHPLC-MS/MS
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Introduction

At the end of renal failure, most patients undergo lifelong hemodialysis (HD) therapy. Although HD techniques have recently been improved, patients may experience several complications with quotidian therapy. HD patients often have muscle cramps, which are defined as the sudden onset of a prolonged involuntary muscle contraction accompanied by severe pain, resulting in early termination of an HD session and inadequate dialysis (Chillar and Desforges 1972). A number of palliative measures are currently employed, but consistent effects have not been obtained (Khajehdehi et al. 2001). Through HD therapy, water-soluble vitamins are indiscriminately removed from plasma together with uremic toxins, and these vitamins tend to be deficient in HD patients. Since biotin, a water-soluble vitamin, plays an essential role as one of the coenzymes of the citric acid cycle in producing energy, we hypothesized that biotin may intervene in the occurrence of HD-associated cramps. Biotin is a B-complex vitamin that plays important biochemical roles, the most important being its involvement in carbon dioxide transfer as a coenzyme for several carboxylases, such as acetyl-CoA, propionyl-CoA, β-methylcrotonyl-CoA, and pyruvate carboxylases. These four enzymes catalyze critical steps in the pathways of intermediary metabolism, including the synthesis of fatty acids, catabolism of branched-chain amino acids, and gluconeogenesis (Zempleni and Mock 1999).

In our previous study (Oguma et al. 2012), oral biotin was administered to patients with frequent muscle cramps during HD sessions. The administered biotin promptly reduced the incidence and severity of cramps in most

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patients. We then measured plasma biotin concentrations in HD patients at baseline (before biotin administration) by an avidin-competitive enzyme-linked immunosorbent assay (ELISA). Unexpectedly, plasma levels of biotin, measured by an ELISA were elevated in HD patients, compared to healthy subjects. In addition, HD patients with cramps had higher levels of plasma biotin than those without cramps. Although biotin in plasma was excessive in HD patients, the administration of biotin ameliorated cramps, suggesting impaired activities of biotin in HD patients with cramps.

We classified patients with cramps into two groups according to the effect of exogenous biotin: patients with a relatively significant effect (well-responders) and patients with little or no effect (poor-responders). The ELISA measurements indicated that the poor-responders tended to have higher biotin levels, whereas well-responders tended to have lower biotin levels.

In fact, the ELISA detected the total avidin-binding substances (TABS) which constitute biotin-backbones, *i.e.* not only biotin and but also its analogues and metabolites (Mock et al. 1995). Biotin metabolites that originate from either β-oxidation, from sulfur oxidation, or both have been identified in mammals. They include bisnorbiotin, teteranorbiotin, biotin sulfoxide and biotin sulfone. All of them retain a heterocyclic ring as biotin in structures; thus, the above-mentioned biotin metabolites are measured as TABS. Therefore, it is necessary to measure biotin *per se* selectively out of TABS in patients.

Mock et al. (1995) measured biotin levels by high-performance liquid chromatography (HPLC) using a C_{18} reversed-phase column followed by ELISA in healthy human plasma. Their method, however, had the drawback of requiring many procedures, and was unsuitable for multiple measurements. Recently, we have successfully developed a simple ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method to quantify plasma biotin of HD patients with sufficient sensitivity (Yagi et al. 2016). In the present study, we applied UHPLC-MS/MS and ELISA to determine the plasma levels of biotin and TABS, respectively, in HD patients and healthy subjects. Previous studies have shown that biotin accounts for only half of TABS in human biofluids (Mock et al. 1995; Bogusiewicz et al. 2008). It is hypothesized that TABS subtracted by biotin are biotin metabolites. In the present study, levels of these biotin metabolites in HD patients and healthy subjects were measured. This may provide new information about the relation between biotin activity and cramps in HD patients.

Materials and Methods

Patients

We defined cramp scores based on the severity and frequency in HD patients. Scores were measured before and after oral biotin administration as reported previously (Oguma et al. 2012). Twenty-eight HD patients (12 patients without cramps and 16 patients with cramps) and 11 healthy volunteers were studied. Their written informed consent was obtained beforehand. For the following analysis, we classified the subjects into four groups: 11 healthy subjects, 12 HD patients without cramps, 7 well-responders to exogenous biotin with cramps, and 9 poor-responders to exogenous biotin with cramps. The study was approved by the Ethics Committee of the Tohoku University Graduate School of Pharmaceutical Sciences (Sendai, Japan) and Koujinkai Hospital (Sendai, Japan).

Sample collection

Blood samples were collected into lithium heparin tubes. The samples from HD patients were taken before biotin administration and pre-HD. After centrifuging, the plasma was stored at −25°C. It was thawed on ice immediately before measurement and any precipitation was removed by centrifugation.

Measurements of plasma biotin concentrations by ELISA and UHPLC-MS/MS

The ELISA measurement of plasma samples was carried out in the same way as previously reported (Oguma et al. 2012) using an ELISA kit (Immundiagnostik, GmbH, Bensheim, Germany) according to the instructions of the maker (Wellenberg and Banks 1993). The lower detection limit of ELISA was 0.015 μg/l. In the new UHPLC-MS/MS method, the necessary detection limit of biotin separately was 0.05 μg/l. In the plasma of HD patients, the pentafluorophenyl stationary phase column was applied to avoid a strong matrix effect and many interference peaks in the biotin region (Yagi et al. 2016). However, with that method we have not yet been able to determine metabolites selectively, such as bisnorbiotin, biotin sulfoxide and biotin sulfone in the plasma of HD patients. We defined the concentration of biotin metabolites as TABS (measured by the ELISA) without biotin (measured by the UHPLC-MS/MS).

Statistical analysis

Statistical analyses were performed with JMP®Pro version 11.0 (SAS Institute Japan Ltd., Tokyo, Japan). Results are presented as median values with upper and lower quartiles for biotin concentrations. Comparisons between groups are given as Wilcoxon rank sum tests for distributions of the concentrations. Differences of $p < 0.05$ are considered to be statistically significant.

Results and Discussion

Backgrounds of subjects

Table 1 shows the backgrounds of the patients in the groups. There are no significant differences in their data of age, sex, and weight. Kt/V is the parameter which indicates the HD efficacy of small uremic-toxin removal (Daugirdas 1993). In which there were no significant differences among the groups, and biotin and its metabolites were filtered out equally. The groups had no significant differences in kinds of dialysate buffers (acetate or citrate). The patients with cramps had significantly longer HD duration than the patients without cramps. Generally, patients with longer period on HD experience several complications due to HD treatments. The mean age of the 11 healthy subjects (5 males) was 57.7 ± 10.2 (range 34 to 69) years old.

Table 1. Baseline characteristics of study patients (n = 28).

	Patients with cramps n = 16			Patients without cramps n = 12	p (cramps vs. non-cramp)
	well-responders n = 7	poor-responders n = 9	p (well vs. poor)		
Age (y)	64 ± 6	64 ± 6	0.89	67 ±13	0.21
HD duration (y)	20 ± 13	18 ± 8	0.65	10 ± 9	*0.03
Males (%)	2 (29)	6 (67)	0.13	7 (58)	0.66
Weight (Kg)	54.8 ± 9.5	56.8 ± 8.0	0.36	52.6 ± 18.1	0.4
Kt/V	1.69 ± 0.35	1.51 ± 0.18	0.42	1.42 ± 0.33	0.89
Dialysate (Ac/Ct) [a]	3 /4	3 / 6	0.7	7/5	0.27

Table shows baseline characteristics of the patients with and without cramps. Patients with cramps are stratified into two groups; a group with well-response (n = 7) and a group with poor-response (n = 9).
Values are "mean ± SD" or "number and percentage". Kt/V is the efficacy indicator of in HD treatment (see in the text).
[a]HD treatments used bicarbonate dialysis buffers (containing 8-12 mM acetate and 5-8 mM glucose) (indicated as Ac), or citrate dialysis buffers (containing 0.7 mM citrate and 8 mM glucose) (indicated as Ct). An asterisk, *, indicates values with $p < 0.05$. The chi-square test was performed to compare categorical variables (sex and dialysate buffer) between the two groups.

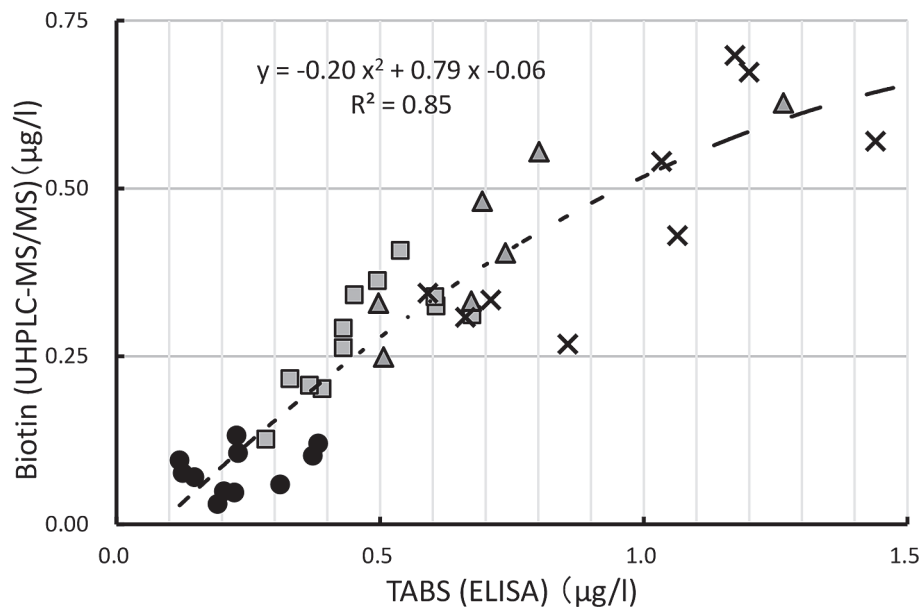

Fig. 1. Correlation between plasma levels of TABS and biotin.
TABS were measured with ELISA (μg/l) and biotin was measured with UHPLC-MS/MS (μg/l).
●: 11 healthy subjects; ■: 12 HD patients without cramps; ▲: 7 HD patients with cramps who responded well to exogenous biotin; ×: 9 HD patients with cramps who responded poorly to exogenous biotin. Data are approximated by the polynomial equation. R^2 is a decision coefficient.

Plasma concentration of TABS and biotin measured by ELISA and UHPLC-MS/MS in each subgroup

A total of 39 plasma samples obtained from healthy subjects and HD patients were measured with both ELISA and UHPLC-MS/MS. The concentration of TABS and biotin are shown in Fig. 1. The relation between TABS and biotin was quadratic, which was fitted as $y = -0.20\ x^2 + 0.79\ x - 0.06$, indicating that the higher the TABS (x) was, the higher the biotin (y) was. As was the case in previous studies (Mock et al. 1995; Bogusiewicz et al. 2008), biotin (y) accounted for half of TABS (x) in the healthy subjects (Fig. 1), while in the HD patients biotin (y) was less than half of TABS (x) (Fig. 1).

Fig. 2a shows a comparison of the levels of TABS

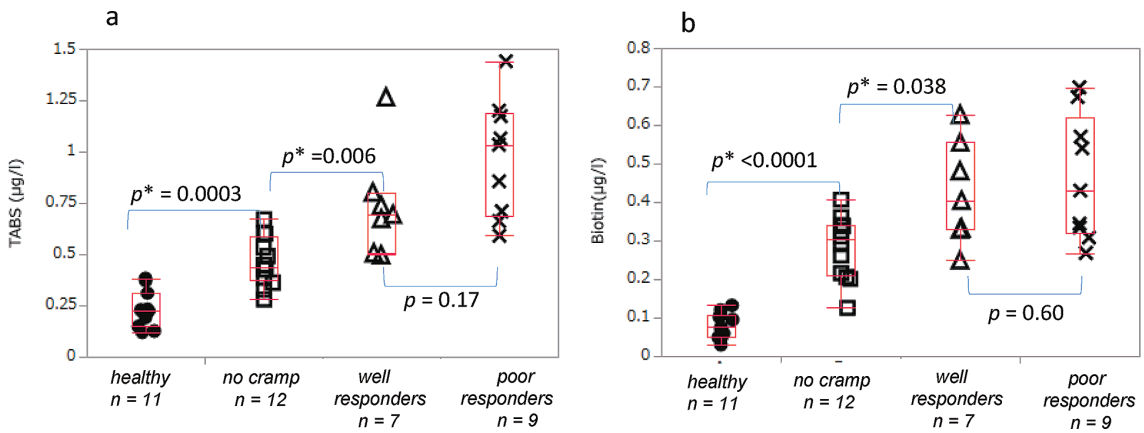

Fig. 2. Box plots of plasma levels of TABS and biotin.
(a) TABS measured with ELISA and (b) biotin measured with UHPLC-MS/MS. In each graph, box plots are appended by median, quartiles, minimum and maximum. Each graph shows a comparison among the four groups: 11 healthy subjects, 12 HD patients without cramps, 7 well-responders, and 9 poor-responders. Between the groups, *p*-values are depicted and statistically significant *p*'s are denoted by asterisks.

measured by an ELISA by the box plots among the four groups. The medians and quartiles of TABS in healthy subjects, in patients without cramps, in well-responders to exogenous biotin and in poor-responders to exogenous biotin were 0.22 (0.15-0.31), 0.44 (0.37-0.59), 0.69 (0.51-0.80) and 1.03 (0.69-1.19) μg/l, respectively. There were significant differences between healthy subjects and patients without cramps (p = 0.0003), and between patients without cramps and well-responders (p = 0.006), whereas there was no significant difference between well-responders and poor-responders (p = 0.17).

Fig. 2b illustrates biotin levels among the four groups measured by the UHPLC-MS/MS. The medians and quartiles of biotin for the four groups were 0.08 (0.05-0.11) for healthy subjects, 0.30 (0.21-0.34) for patients without cramps, 0.40 (0.33-0.56) for well-responders, and 0.43 (0.32-0.62) μg/l for poor-responders, indicating that biotin accumulated in HD patients. HD patients without cramps had significantly higher levels (p < 0.0001) than healthy subjects. Biotin had significantly higher levels in well-responders than HD patients without cramps (p = 0.038). Poor-responders had only slightly higher levels of biotin than well-responders, and the difference was not significant (p = 0.60). It should be noted that no deficiency in biotin was exhibited in HD patients, and even HD patients with cramps had abundant biotin (Fig. 2b).

Estimation of biotin metabolites

The plasma levels of biotin metabolites, expressed as TABS subtracted by biotin, among the four groups are illustrated in Fig. 3. The medians and quartiles of the concentrations in the four groups were 0.16 (0.08-0.25) for healthy subjects, 0.16 (0.13-0.25) for patients without cramps, 0.26 (0.21-0.34) for well-responders and 0.49 (0.37-0.61) μg/l for poor-responders. HD patients without cramps had slightly higher levels of biotin metabolites than controls, but without significant difference (p = 0.34). Significant differences in biotin metabolites were observed between HD patients without cramps and well-responders with cramps (p = 0.025).

Of particular note is that poor-responders had significantly higher metabolites than well-responders (p = 0.039), despite the finding that poor- and well-responders had no significant differences in TABS and biotin (Fig. 2a, b). To sum up, the distributions of the concentrations of TABS, biotin, and metabolites among the four groups all had a common trend, *i.e.*, healthy subjects < patients without cramps < well-responders < poor-responders (Figs. 2 and 3).

As was previously reported, TABS are not only biotin but also its metabolites such as bisnorbiotin and biotin sulfoxide, which have no activity as coenzymes (Mock et al. 1995; Zempleni et al. 1997; Bogusiewicz et al. 2008) It is possible that these metabolites compete and interfere with the effect of active biotin for intestinal or cellular uptake, or delivery to tissues (Said 1999). In patients with cramps, biotin metabolites may have interfered with the activity of biotin, even though biotin was excessive in HD patients. This effect of biotin metabolites may cause cramps. Exogenous biotin may overcome the interfering effect of accumulated biotin metabolites in well-responders. If higher doses of biotin are administered, cramps may be ameliorated even in the poor-responders. The present results suggest a possible action of biotin, which intervenes in the mechanism of muscle cramps during or after HD. Biotin may compensate for subnormal energy metabolism in HD patients.

In conclusion, the present study has demonstrated that the plasma concentration of biotin is higher in HD patients with cramps. Moreover, the plasma concentration of biotin metabolites is significantly higher in poor-responders than in well-responders to exogenous biotin. Biotin metabolites may impair biotin's functions as a coenzyme.

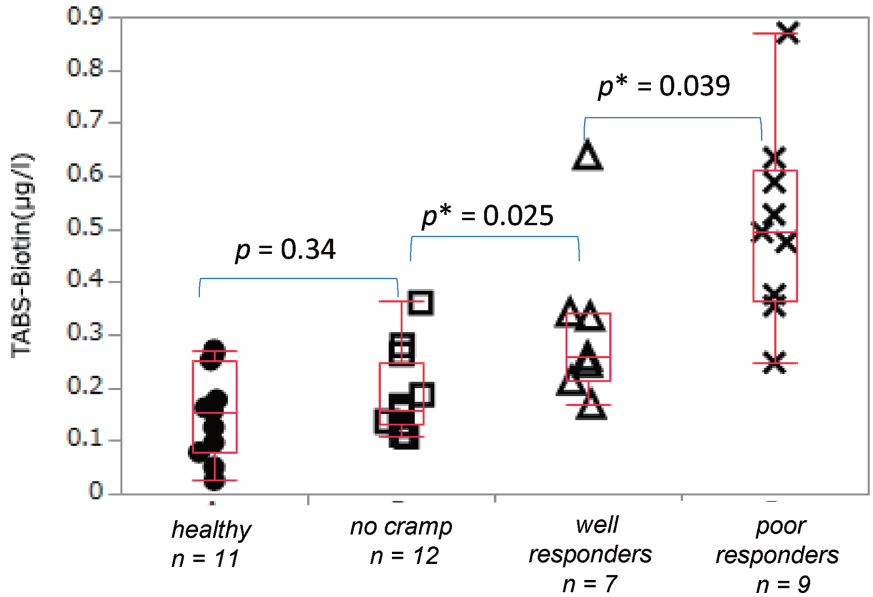

Fig. 3. Box plots of the plasma levels of biotin metabolites (TABS subtracted by biotin).
The box plots are appended by median, quartiles, minimum and maximum. The graph shows a comparison among the four groups: 11 healthy subjects, 12 HD patients without cramps, 7 well-responders, and 9 poor-responders. Between the groups, *p*-values are depicted and statistically significant *p*'s are denoted by asterisks.

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Conflict of Interest

The authors declare no conflict of interest.

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