Bull. Environ. Contam. Toxicol. (1988) 40:553–560


Environmental Contamination and Toxicology

# Studies on the Increase in Serum Concentrations of Urea Cycle Amino Acids among Subjects Exposed to Cadmium

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Itai-itai disease (I disease) is a combination of renal tubular damage and osteomalacia accompanied by osteoporosis among subjects exposed to cadmium (Cd). When the renal tubular damage progresses, the excretion of amino acids, especially, threonine, hydroxyproline, proline, citrulline, ornithine, arginine etc. increased in urine (Fukushima et al 1974; Hoshino et al 1975; Nishino et al 1978; Nogawa et al 1980; Kobayashi et al 1982). Hoshino et al (1975) reported that the increase in urinary excretion of citrulline, arginine and ornithine may be associated with an inhibition of urea synthesis in the urea cycle. We have found that serum citrulline, arginine and ornithine also increased in I disease patients (Nishino et al 1980). In order to investigate the mechanism of the increase in these serum amino acids, comparative studies were performed using both healthy subjects and patients with renal disease as control groups.

## MATERIALS AND METHODS

The study group (Cd-exposed group) consisted of 11 female I disease patients and 13 female so-called Itai-itai disease observation patients (Observation patients, Shiroishi et al 1977) with renotubular damage and without typical bone disorder. The mean ages were 73 years (60-86 years) and 72 years (59-83 years), respectively. The control group with renal diseases (Renal disease patients not related to Cd) consisted of 7 patients with chronic glomerulonephritis, 3 with nephrosis and 5 with chronic renal failure receiving no dialysis treatment. The mean age of 9 males and 6 females of this group was 43 years (24-70 years). The healthy control group (Healthy control 2) consisted of 22 males and 27 females. The mean ages were 47 years (21-80 years) and 54 years (25-86 years), respectively. Of the Healthy control 2, subjects matched in sex and age for the Cd-exposed ones were reassigned to another healthy control (Healthy control 1). No subject belonging to the Renal disease patients and the Healthy controls had a history of living in Cd-polluted areas.

The blood samples were drawn within two hours after breakfast (Milsom et al 1979). Twenty-four-hour urinary specimens were collected from both the Cd-exposed group and the Healthy controls,

and one-hour urinary specimens were collected from the Renal disease patients. In order to determine creatinine clearance (Ccr) and tubular reabsorption of phosphorus (% TRP) for the Renal disease patients, the mean concentration of 2 urinary specimens collected at one-hour intervals was used. After the deproteinization of serum (precipitation in a final concentration of 3% sulfosalicylic acid, Hamilton et al 1962) and urine (ultrafiltration through UM-2 membrane), the determination of amino acid concentrations was performed by Hitachi Liquid Chromatography type 034. Li-buffer solutions (pH3.18, pH3.70) were used for the measurement of neutral and acidic amino acid concentrations and Na-buffer solutions (pH3.4, pH4.8) for basic amino acids. Creatinine, inorganic phosphorus, urea and ammonium were determined by Jaffe method (Jaffe 1886), Fiske-Subba Row method (Fiske and SubbaRow 1925) and liquid chromatography, respectively. Statistical analysis of results were performed by t-test.

## RESULTS AND DISCUSSION

The values of parameters for glomerular (Ccr) and tubular (% TRP) functions are shown in Table 1. The Ccrs in the I disease patients and the Observation patients were significantly lower than that in the Healthy control 1 ($p < 0.001$). The same was true for the Renal disease patients compared with the Healthy control 2 ($p < 0.001$). With regard to % TRP, similar kinds of differences were observed for Ccr ($p < 0.001$). Since no differences were observed in Ccr or % TRP between the I disease patients and the Observation patients, these two groups were designated as the Cd-exposed group. In comparing the Cd-exposed group with the Renal disease patients, there was no significant difference in Ccr but a significant difference was observed in % TRP. The Cd-exposed group showed a lower % TRP than the Renal disease patients ($p < 0.01$).

The values of serum concentration of citrulline, arginine and ornithine are shown in Table 2. Serum citrulline concentrations in the Cd-exposed group and the Renal disease patients were more than two times higher than that in either Healthy control 1 or 2 ($p < 0.001$, $p < 0.01$, respectively). In addition to an increase in serum citrulline concentration of uremic patients (Condon and Asatoor 1971; Chan 1974), we have demonstrated that serum concentrations of creatinine, urea and Ccr as the parameters of glomerular functions were significantly correlated with serum citrulline concentration ($r=0.67$, $p < 0.01$; $r=0.60$, $p < 0.05$; $r=-0.53$, $p < 0.05$; respectively) but % TRP was not. Thus, these results indicated that an increase in serum citrulline concentration is associated with glomerular dysfunction. In the Cd-exposed group, there was no significant correlation between the serum citrulline concentration and the parameters mentioned above. However, in the subjects with relatively moderate glomerular dysfunction (Ccr > 20 ml/min) in the Cd-exposed group, a significant correlation was noticed between serum citrulline concentration and Ccr ($r=-0.72$, $p < 0.05$) but not between serum citrulline concentration and % TRP. In Table 3, serum amino acid concentrations in the Cd-

Table 1. Renal functions in Itai-itai disease, Itai-itai disease observation and renal disease patients (Mean ± S.D.)

| | Cd-exposed group | | Non-Cd-exposed group | | |
|---|---|---|---|---|---|
| | Itai-itai disease patients | Observation patients | Renal disease patients | Healthy control 1 | Healthy control 2 |
| n | 11 | 13 | 15 | 14 | 24 |
| sex | Women | Women | Men & Women | Women | Men & Women |
| Age | 73 ± 8 | 72 ± 7 | 43 ± 15 | 74 ± 8 | 56 ± 23 |
| Ccr (ml/min) | 19 *1) ± 12 | 21 *1) ± 11 | 31 *2) ± 21 | 57 ± 22 | 84 ± 38 |
| % TRP | 49 *1) ± 15 | 41 *1) ± 16 | 68 *2) ± 10 | 82 ± 3 | 82 ± 5 |

1) Compared with Healthy control 1
2) Compared with Healthy control 2 * $p < 0.001$

Table 2. Serum concentrations of urea cycle amino acids (μ mole/ml)

| | Cd-exposed group | | Renal disease patients | | Healthy control 1 | | Healthy control 2 | |
|---|---|---|---|---|---|---|---|---|
| | n | Mean ± S.D. | n | Mean ± S.D. | n | Mean ± S.D. | n | Mean ± S.D. |
| Citrulline | 24 | 0.064 ***1) ± 0.026 | 15 | 0.053 **2) ± 0.035 | 14 | 0.027 ± 0.012 | 49 | 0.026 ± 0.013 |
| Arginine | 23 | 0.139 ***1) ± 0.045 | 15 | 0.105 ns2) ± 0.053 | 14 | 0.074 ± 0.019 | 47 | 0.082 ± 0.040 |
| Ornithine | 23 | 0.105 ns1) ± 0.030 | 15 | 0.070 *2) ± 0.017 | 14 | 0.091 ± 0.033 | 47 | 0.084 ± 0.031 |

1) Compared with healthy control 1
2) Compared with healthy control 2
* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$
ns; not significant

Table 3. Serum concentrations of urea cycle amino acids by creatinine clearance level (μ mole/ml)

| | | Cd-exposed group | | Renal disease patients | | Difference between two groups (t-test) |
|---|---|---|---|---|---|---|
| | | n | Mean ± S.D. | n | Mean ± S.D. | |
| Citrulline | Ccr ≦ 20 ml/min | 13 | 0.058 **1) ± 0.024 | 6 | 0.074 *2) ± 0.044 | |
| | Ccr > 20 ml/min | 11 | 0.070 ***1) ± 0.030 | 9 | 0.039 ± 0.020 | * |
| Arginine | Ccr ≦ 20 ml/min | 13 | 0.135 ***1) ± 0.042 | 6 | 0.153 ***2) ± 0.044 | |
| | Ccr > 20 ml/min | 10 | 0.145 ***1) ± 0.051 | 9 | 0.074 ± 0.028 | ** |
| Ornithine | Ccr ≦ 20 ml/min | 13 | 0.095 ± 0.014 | 6 | 0.071 ± 0.019 | ** |
| | Ccr > 20 ml/min | 10 | 0.108 ± 0.033 | 9 | 0.069 ± 0.017 | ** |

1) Compared with healthy control 1
2) Compared with healthy control 2
* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

exposed group and in the Renal disease patients are shown by the Ccr level. In the Cd-exposed group, the serum citrulline concentration was significantly higher at both Ccr levels compared with the Healthy control (Ccr $\leqq$ 20 ml/min, $p < 0.01$; Ccr > 20 ml/min, $p < 0.001$; respectively). On the other hand, in the Renal disease patients such a significant difference ($p < 0.05$) was observed only for the subgroup with relatively severe glomerular dysfunction (Ccr $\leqq$ 20 ml/min). In addition, different mechanisms are suggested for the increased serum citrulline concentration between the two groups, since the serum citrulline concentration was higher in a subgroup of the Cd-exposed group than in the counterpart subgroup of the Renal disease patients.

Serum arginine concentration in the Cd-exposed group was significantly higher than that in the Healthy control 1 ($p < 0.001$) but in the Renal disease patients it was not different from that in the Healthy control 2 (Table 2). The finding in the Renal disease patients is consistent with the report of Kopple and Jones (1979). However, serum arginine concentration in the Renal disease patients was significantly correlated with serum concentration of creatinine ($r=0.87$, $p < 0.001$), urea ($r=0.84$, $p < 0.001$) and Ccr ($r=-0.69$, $p < 0.01$). Furthermore, when the Renal disease patients were divided into 2 subgroups by Ccr levels, the subgroup with Ccr $\leqq$ 20 ml/min showed significantly higher serum arginine concentration compared with the Healthy control 2 ($p < 0.001$, Table 3). These results suggested that serum arginine concentration in the Renal disease patients was also associated with glomerular dysfunction as was serum citrulline concentration. In fact, serum arginine concentration was significantly correlated with serum citrulline concentration ($r=0.60$, $p < 0.05$) in the Renal disease patients. For the Cd-exposed group, serum arginine concentration showed no significant correlation ($r=0.15$ for creatinine , $r=0.05$ for urea and $r=-0.16$ for Ccr, respectively) with the parameters of glomerular and tubular functions mentioned above, though the concentrations in both subgroups classified by Ccr level were significantly higher than that in the Healthy control 1 ($p < 0.001$, respectively, Table 3). The significant correlation between serum arginine and citrulline concentration which was confirmed in the Renal disease patients was not observed in the Cd-exposed group. As for the Cd-exposed group, as in the case of an increase in serum citrulline concentration, the increased serum arginine concentration may be due to a different mechanism from that in the Renal disease patients.

Serum ornithine concentration in the Renal disease patients was significantly lower than that in the Healthy control 2 ($p < 0.05$) but in the Cd-exposed group it was not different from that in the Healthy control 1 (Table 2). In either the Renal disease patients or the Cd-exposed group, no significant correlation was observed between serum ornithine concentration and the parameters of glomerular and tubular functions mentioned above. Furthermore, there was no significant difference in the serum ornithine concentration between the two subgroups classified by Ccr level in the

Table 4. Urinary excretion rate of urea cycle amino acids (μ mole/ml)

| | Cd-exposed group | | Renal disease patients | | Healthy control 1 | | Healthy control 2 | |
|---|---|---|---|---|---|---|---|---|
| | n[1] | G.M.[2] (S.D.[3]) | n | G.M. (S.D.) | n | G.M. (S.D) | n | G.M. (S.D.) |
| Citrulline | 24/24 | 0.272[***4] (2.249) | 11/15 | 0.013 (2.701) | 8/14 | 0.008 (1.600) | 9/24 | 0.009 (1.875) |
| Arginine | 24/24 | 0.124[***4] (2.125) | 13/15 | 0.009 (2.747) | 10/14 | 0.013 (1.788) | 16/24 | 0.014 (1.694) |
| Ornithine | 24/24 | 0.141[***4] (3.841) | 14/15 | 0.019 (4.749) | 7/14 | 0.013 (3.162) | 14/24 | 0.020 (3.601) |

1) Number of samples in which urea cycle amino acids were detected/number of samples examined
2) Geometric mean 3) Geometric standard deviation
4) Compared with healthy control *** $p < 0.001$

Table 5. Urinary excretion rate of urea cycle amino acids at a creatinine clearance level (μ mole/ml)

| | | Cd-exposed group | | Renal disease patients | | Difference between 2 groups |
|---|---|---|---|---|---|---|
| | | n[1] | G.M.[2] (S.D.[3]) | n | G.M. (S.D.) | |
| Citrulline | Ccr ≦ 20 ml/min | 13/13 | 0.173 (1.862) | 5/6 | 0.008 (1.748) | *** |
| | Ccr > 20 ml/min | 11/11 | 0.465[**4] (1.985) | 6/9 | 0.022 (3.388) | *** |
| Arginine | Ccr ≦ 20 ml/min | 13/13 | 0.104 (2.095) | 6/6 | 0.005 (2.148) | *** |
| | Ccr > 20 ml/min | 11/11 | 0.160 (2.093) | 7/9 | 0.014 (2.764) | *** |
| Ornithine | Ccr ≦ 20 ml/min | 13/13 | 0.051 (7.617) | 6/6 | 0.012 (5.963) | ** |
| | Ccr > 20 ml/min | 11/11 | 0.233[*4] (1.900) | 8/9 | 0.027 (3.987) | *** |

1) Number of samples in which urea cycle amino acids were detected/number of samples examined
2) Geometric mean 3) Geometric standard deviation
4) Compared with Ccr ≦ 20 ml/min group
* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Renal disease patients and in the Cd-exposed group, though it was significantly lower in the former than in the latter when compared between counterpart subgroups ($p < 0.01$, respectively, Table 3). Taken together, the findings concerning the serum concentrations of urea cycle amino acids indicate that ornithine behaves differently from citrulline and arginine in the serum.

The values of urinary excretion rate (μ mole/min) of citrulline, arginine and ornithine are shown in Table 4. The values of the three amino acids in the Cd-exposed group were significantly higher than those in the Healthy control 1 ($p < 0.001$), but in the

Renal disease patients they were not different from those in the Healthy control 2 (Table 4). With regard to the relationship between urinary excretion rate of citrulline and % TRP, the subgroup with Ccr > 20 ml/min of the Cd-exposed group showed a significant correlation ($r=-0.72$, $p < 0.05$) but the subgroup with Ccr $\leqq$ 20 ml/min did not. On the other hand, urinary excretion rate of citrulline in the latter subgroup was significantly lower than that of the former subgroup ($p < 0.01$, Table 5).
Identical results were observed for ornithine in the Cd-exposed group; that is, a significant correlation ($-0.61$, $p < 0.05$) in the subgroup with Ccr > 20 ml/min and the significantly lower urinary excretion rate in the subgroup with Ccr $\leqq$ 20 ml/min ($p < 0.05$, Table 5) were observed. These results indicate that amino-acid uria in the subgroup with Ccr > 20 ml/min mainly reflects damage to tubular reabsorption. However, similar significant correlations were not observed for arginine in the Cd-exposed group.
The disappearance of a significant correlation between urinary excretion rate and % TRP in the subgroup with Ccr $\leqq$ 20 ml/min suggests that a reduced filtration of amino acids due to severe glomerular damage overcomes a reduced % TRP. On the other hand, in the Renal disease patients urinary excretion rates of amino acids were not correlated with % TRP. Furthermore, the urinary excretion rate was not different between the two subgroups classified by Ccr level, being at the same level as that of the Healthy control 2. These findings indicate that in the Renal disease patients amino acids can be reabsorbed due to an almost intact tubular function.

As well known, citrulline is formed by the combination of ammonia with ornithine in the liver, and subsequently converted to arginine, from which urea and ornithine are produced in the liver and kidney. Since the increases in serum concentrations of urea etc. due to renal dysfunction have been demonstrated to cause reduced production of urea etc. (Merrill, 1949; Goldman, 1954), the activity of arginase contributing to urea production seems to be suppressed in the renal disease. In addition, a considerable increase in plasma concentration of arginine and a decrease in that of ornithine were reported in patients with arginase deficiency (Kang et al 1983). Hence, our findings suggest that the markedly increased serum concentration of urea in the Renal disease patients may have caused an increase in serum arginine concentration and a decrease in serum ornithine concentration due to suppression of arginase activity. This process also might be the case in the Cd-exposed group with the increased serum urea concentration. Chan et al (1974) have reported an increased citrulline concentration in the plasma, liver and muscle of uremic rats, and a decreased arginine synthetase activity of kidney accompanied with normal activity of arginino-succinate synthetase and arginino-succinase of liver.
Taking into consideration the above mentioned results, it is suggested that the increased serum concentration of citrulline in the Renal disease patients may be due to the suppressed conversion of citrulline to arginine in the kidney. The identical change in serum concentration of citrulline may be also expected in the Cd-

exposed group with advanced glomerular dysfunction. In fact this was the case in the present study.
In addition to the mechanisms discussed above, we would also like to take into consideration the Cd-induced effects on urea cycle enzymes. Suzuki et al (1980) have reported a suppression in argininosuccinase activity in the liver of Cd-fed mice. However, they were unable to demonstrate an increase in urinary amino acids. These findings indicate that the suppression is more closely associated with Cd-induced effects on urea cycle enzymes of liver rather than those of kidney.

In summary, the increased serum concentrations of urea cycle amino acids in the Cd-exposed subgroup with moderate glomerular dysfunction might be due not only to reduced renal functions but also to Cd-induced effects on urea cycle enzymes of liver. Therefore, we would like to propose that patho-biochemical mechanisms of the increased serum concentrations of urea cycle amino acids are different in the Cd-exposed subjects from those in the unexposed patients with renal diseases.

Acknowledgments. We would like to thank Dr. H. Uetake, Director of Toyama Institute of Health, for helpful suggestions during the process of this research, and Miss F. Sakai, Department of Community Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, for her help in preparation of this manuscript.

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Received June 18, 1987; accepted January 26, 1988