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Relationship between the level of serum L-tryptophan and its hepatic uptake and metabolism in rats with carbon tetrachloride-induced liver cirrhosis

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Summary. In the serum of rats with liver cirrhosis induced by 12-week intermittent carbon tetrachloride (CCl_4) injection, free L-tryptophan (Trp) levels increased with decreases in total Trp, albumin-bound Trp, and albumin levels. In the serum of the cirrhotic rats, there were no changes in the ratio of albumin-bound Trp to albumin and the level of free fatty acids which are known to weaken the binding of Trp to albumin. In the liver of the cirrhotic rats, there were increases in protein and free Trp (i.e., non-protein Trp) contents and a decrease in total tryptophan 2,3-dioxygenase (TDO) activity. The decreased TDO activity was mainly due to the reduction of apo-TDO activity. When [^{3}H]Trp was injected into the portal vein of the cirrhotic and control rats, radioactivity derived from the injected [^{3}H]Trp in the liver was higher in the cirrhotic rats than in the control rats at 10 min after the injection, while the radioactivity in the serum was lower in the former rats than in the latter rats. These results indicate that the increased Trp is easily taken up into the cirrhotic liver, and suggest that the Trp taken up into the cirrhotic liver could be utilized for the maintenance of synthesis of proteins in the tissue through the reduction of Trp metabolism due to reduced TDO activity in the tissue.

Keywords: L-Tryptophan – Transport – Metabolism – Liver – Carbon tetrachloride – Experimental liver cirrhosis (rat)

Introduction

Under physiological conditions, 80–90% of L-tryptophan (Trp), one of the essential amino acids, in the blood plasma or serum of animals and humans exists in an albumin-bound form and the remainder (ca. 10μM) is present in an albumin-unbound form, i.e., a free form (McMenamy et al., 1957; McMenamy and Oncley, 1958; Fuller and Roush, 1973; Saito et al., 1986). The binding of Trp to albumin is affected not only by the concentration of albumin

itself but also by long-chain fatty acids which are known to bind to albumin more strongly than Trp (Curzon et al., 1973; Curzon et al., 1974; Brodersen et al., 1989; Sasaki et al., 1993). Under physiological conditions, more than 90% of Trp present in the plasma is metabolized in the liver (Bender, 1982). In the liver, Trp is metabolized to kynurenine and subsequently to acetyl CoA and NAD via the kynurenine pathway in which tryptophan 2,3-dioxygenase (TDO) participates not only as the first metabolizing enzyme but also as the rate-limiting enzyme, in addition to its utlilization for protein synthesis (Bender, 1982). Trp is known to be taken up into liver cells via the transport systems common to aromatic amino acids such as L-phenylalanine and L-tyrosine and branched-chain amino acids such as L-leucine, L-isoleucine, and L-valine (Saito et al., 1986; Salter et al., 1986). In addition, it has been shown that Trp is taken up into isolated rat hepatocytes via at least two saturable transport systems (one high-affinity and one low-affinity) and one non-saturable transport system, and that the high-affinity and low-affinity transport systems possess the Km values of 2.4 μM and 2.1 mM, respectively (Saito et al., 1986).

It has been reported that in the plasma or serum of patients with liver cirrhosis, total Trp levels tend to decrease with an apparent increase in free Trp levels (Cangiano et al., 1976; Yoshida and Hirayama, 1980; Zoli et al., 1981; Hijita et al., 1981; Rocchi et al., 1986). Zoli et al. (1981) have shown using the plasma of normal subjects and cirrhotic patients that the amount of albumin-bound Trp directly correlates with the level of albumin, and to a lesser extent with the level of free fatty acids (FFA), but not with the level of bilirubin. It has been reported that in patients with liver cirrhosis, albumin synthesis is normal or elevated, although serum albumin levels are depressed (Hasch et al., 1967; Rothschild et al., 1969b). It has also been shown in rats with liver cirrhosis induced by chronic carbon tetrachloride (CCl_4) treatment that albumin synthesis is increased (Huberman and Soberon, 1970; Kershenobich and Rojkind, 1973), that albumin gene transcription is rather enhanced (Panduro et al., 1988), and that hepatic protein synthesis is maintained at the normal level (Sidransky et al., 1988). It is known that Trp is the limiting amino acid for albumin synthesis under some circumstances (Rothschild et al., 1969a). It is also known that Trp itself has a stimulating effect on liver protein synthesis by enhancing both m-RNA synthesis and its translocation from the nucleus to the cytoplasm in rats and mice (Sidransky, 1985). From these findings, it can be supposed that in liver cirrhosis, increased free Trp in the serum is taken up by the cirrhotic liver and subsequently is utilized for synthesis of proteins such as albumin in the tissue. However, there is no information on these matters at present. We have already demonstrated in rats with a single CCl_4 treatment that in acute liver injury with impaired hepatic Trp uptake via its high-affinity system, an increase in the level of free serum Trp should aid its transport into the liver, leading to the stimulation of protein synthesis in the tissue (Uemura et al., 1989).

We, therefore, examined the relationship between the level of serum Trp and its hepatic uptake and metabolism in rats with liver cirrhosis induced by long-term intermittent CCl_4 treatment.

Materials and methods

Male Wistar rats aged six weeks were purchased from SLC Co. (Hamamatsu, Japan). The animals were housed in stainless steel cages in a controlled room at 25°C and 50% humidity on a 12h:12h dark-light cycle and given standard rat chow, Oriental MF (Oriental Yeast Co., Tokyo, Japan), and tap water *ad libitum* throughout this study. Rats were injected subcutaneously with a 50% (v/v) CCl_4 solution in olive oil at a dose of 0.5ml/kg body weight (B.W.) twice a week over a 12 week period. The control rats were given subcutaneously with an equal volume of olive oil twice a week for the same period. These animals were sacrificed by cutting the vena cava caudalis under ether anesthesia at which time blood was collected. The collected blood was separated into serum. Immediately after the sacrifice, livers were isolated, washed with ice-cold 0.15M KCl, and then frozen on dry ice. The frozen livers and serum were stored at −80°C until use.

Serum was use for determinations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin, FFA, and Trp (total and free form). AST and ALT activities in the serum were measured using a commercial kit of Iatrozyme TA-L_Q (Dia-Iatron Co., Tokyo, Japan). These activities are expressed as an international unit (mU/ml). Total protein and albumin in the serum were determined by the biuret method (Gornal et al., 1949) and the bromcresol green method (Doumas et al., 1971), respectively. FFA in the serum were measured using a commercial kit of NEFA KAINOS (Kainos Co., Tokyo, Japan). Trp in the serum was determined using high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Saito et al., 1986; Uemura et al., 1989; Sasaki et al., 1993). Samples for determinations of total Trp and free Trp were prepared as follows: 10min after mixing the serum with an equal volume of ice-cold 0.4M perchloric acid, the mixture was centrifuged at 4°C and 10,000 × g for 10min. The resultant supernatant was used for determination of total Trp. After centrifugation of another aliquot of serum at 4°C and 1,500 × g for 30min in a Centriflo membrane cone CF 25A (Amicon Grace Co., Tokyo, Japan), the resulting filtrate was used for determination of free Trp. The concentration of albumin-bound Trp was estimated from the difference between total Trp and free Trp concentrations determined.

Livers were used for assays of free Trp (i.e., non-protein Trp), protein, total hydroxyproline, and TDO. Free Trp in the liver was determined by the HPLC method described above. The sample for this determination was prepared as follows: the liver was homogenized with 9 volumes of ice-cold 0.4M perchloric acid and the homogenate was centrifuged at 4°C and 10,000 × g for 10min. The resulting supernatant was used for Trp determination. For assays of protein and TDO in the liver, the tissue was homogenized with 9 volumes of ice-cold 0.15M KCl. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. TDO was assayed in the presence or absence of 2μM haematin at 37°C under agitation according to the method of Knox et al. (1966). The activity of apo-TDO was estimated from the difference between the total activity of TDO, obtained in the presence of haematin, and the activity of holo-TDO, obtained in the absence of haematin. One unit of this activity is expressed as the amount of enzyme producing 1μmol kynurenine per h. For determination of total hydroxyproline in the liver, the homogenate prepared for protein and TDO assays was hydrolyzed in 6M HCl at 110°C for 20h. Hydroxyproline was determined by the method of Bondjers and Björkerud (1973).

The transport of Trp into the liver was examined as follows: [^{3}H]Trp (specific activity, 740GBq/mmol), which was purchased from Du Pont/NEN Research Products (Daiichi Pure Chemical Co., Tokyo, Japan), was injected into the portal vein of rats with and without 12-week intermittent CCl_4 injection at a dose of 0.39KBq/kg B. W. under ether anesthesia. Ten min later, the animals were sacrificed by cutting the vena cava caudalis at which time blood was collected and livers were isolated and perfused with ice-cold 0.15M KCl to remove blood remaining in the tissue as much as possible. The collected blood was separated into serum. Radioactivity derived from [^{3}H]Trp injected in the serum and liver was determined as described in our previous report (Sasaki et al., 1993).

All values obtained are expressed as means ± SD. Results were statistically analyzed by Student's *t*-test. The level of significance was set at $p < 0.05$.

Results

When rats received intermittent CCl_4 injection over a 12 week period, body weight in the CCl_4-treated group was significantly lighter than that in the control group without CCl_4 injection, while liver weight in the former group was significantly heavier than that in the latter group on the basis of weight itself and a relative weight, i.e., gram per 100g B.W. (Table 1). Thus, there were apparent changes in both body and liver weights in rats with 12-week intermittent CCl_4 treatment. Therefore, all values of liver components determined below were assessed on the basis of a relative tissue weight.

AST and ALT activities in the serum of rats treated with CCl_4 over a 12 week period were significantly higher than those in the control rats; serum AST and ALT activities in the CCl_4-treated group were 7.2- and 5.0-fold, respectively, higher than those in the control group (Table 2). Total protein and albumin concentrations in the serum of the CCl_4-treated group were significantly lower than those in the control group, while there was no difference in serum FFA concentration between both groups (Table 2).

Table 1. Body and liver weights in CCl_4-treated and control rats

	Control	CCl_4-treated
Body weight (g)	461.0 ± 15.2[1]	345.0 ± 46.1**
Liver weight (g)	14.2 ± 0.4	18.9 ± 3.0*
(g/100g B.W.)	3.04 ± 0.06	5.47 ± 0.36**

[1] Each value is a mean ± SD (n = 5); *$p < 0.01$; **$p < 0.001$ (vs. control).

Table 2. Aspartate aminotransferase (*AST*) and alanine aminotransferase (*ALT*) activities and total protein, albumin, and free fatty acids (*FFA*) levels in the serum of CCl_4-treated and control rats

	Control	CCl_4-treated
AST (mU/ml)	84 ± 9[1]	601 ± 200**
ALT (mU/ml)	29 ± 3	144 ± 19**
Total protein (g/dl)	6.71 ± 0.17	5.87 ± 0.51*
Albumin (g/dl)	4.32 ± 0.29	2.96 ± 0.44**
FFA (μEq/l)	492 ± 154	423 ± 87

AST and ALT activities and FFA in serum were determined using commercial test kits and total protein and albumin in serum were determined as described under Materials and methods.

[1] Each value is a mean ± SD (n = 5); *$p < 0.01$; **$p < 0.001$ (vs. control).

When total Trp, free Trp, and albumin-bound Trp concentrations were examined in the serum of rats with and without 12 weeks of intermittent CCl_4 injection, total Trp and albumin-bound Trp concentrations in the CCl_4-treated rats were significantly lower than those in control rats without CCl_4 treatment, while free Trp concentration in the former group was significantly higher than that in the latter group (Table 3). In addition, the ratio of albumin-bound Trp to total Trp in the CCl_4-treated group was significantly lower than that in the control group (Table 3). There was no difference in the ratio of albumin-bound Trp to albumin, which represents the nmoles of albumin-bound Trp by one gram of albumin, between the cirrhotic and control groups (Table 3).

When the contents of total hydroxyproline, protein, and free Trp, i.e., non-protein Trp were examined in the liver of rats with and without 12-week intermittent CCl_4 injection, the results shown in Table 4 were obtained. The

Table 3. Total L-tryptophan (*Trp*), free Trp, and albumin-bound Trp levels, and the ratio of albumin-bound Trp to either total Trp or albumin in the serum of CCl_4-treated and control rats

	Control	CCl_4-treated
Total Trp (nmol/ml)	129.7 ± 8.8[1]	96.5 ± 13.6*
Free Trp (nmol/ml)	8.8 ± 1.4	12.8 ± 1.7*
Bound Trp (nmol/ml)	120.9 ± 7.7	83.7 ± 0.9**
Bound Trp/total Trp (%)	93.2 ± 0.8	86.5 ± 0.9**
Bound Trp/albumin (nmol/g)	2.80 ± 0.18	2.83 ± 0.41

Total serum Trp and free serum Trp which was separated by centrifugation through an ultrafiltration membrane were determined using high-performance liquid chromatography as described under Materials and methods. The concentration of bound serum Trp was estimated from the difference between total Trp and free Trp concentrations determined.
[1]Each value is a mean ± SD (n = 5). *$p < 0.01$; **$p < 0.001$ (vs. control).

Table 4. Contents of total hydroxyproline, protein, and free Trp in the liver of CCl_4-treated and control rats

	Control	CCl_4-treated
Total hydroxyproline (μmol/liver per 100g B.W.)	5.9 ± 1.1[1]	44.4 ± 3.8**
Protein (g/liver per 100g B.W.)	0.74 ± 0.06	1.20 ± 0.06*
Free Trp (nmol/liver per 100g B.W.)	203.5 ± 43.5	517.2 ± 121.1**

After hydrolysis of liver homogenates in 6M HCl at 110°C for 20h, total hydroxyproline in the hydrolyzed sample was determined. Protein was measured using liver homogenates. After treatment of liver homogenates with 0.4M perchloric acid, free Trp (non-protein Trp) in the deproteinized sample was determined. These measurements were carried out as described under Materials and methods.
[1]Each value is a mean ± SD (n = 5). *$p < 0.01$; **$p < 0.001$ (vs. control).

Table 5. Tryptophan-2,3-dioxygenase (*TDO*) activity in the liver of CCl_4-treated and control rats

	Control	CCl_4-treated
Total TDO activity (units/liver per 100g B.W.)	9.79 ± 1.64[1]	5.57 ± 0.93*
Holo-TDO activity (units/liver per 100g B.W.)	4.50 ± 1.50	3.71 ± 0.64
Apo-TDO activity (units/liver per 100g B.W.)	5.29 ± 1.29	1.86 ± 0.36*

Total and holo-TDO activities in liver homogenates were determined in the presence and absence, respectively, of added haematin as described under Materials and methods, and apo-TDO activity in the homogenate was obtained as the difference of these activities.
[1]Each value is a mean ± SD ($n = 5$); *$p < 0.01$ (vs. control).

Table 6. Radioactivity in the liver and serum of CCl_4-treated and control rats at 10min after injection of [^{3}H]Trp

	Control	CCl_4-treated
Liver (dpm × 10^{-5}/liver per 100g B.W.)	3.94 ± 0.44[1]	8.19 ± 1.23**
Serum (dpm × 10^{-5}/ml)	1.39 ± 0.08	1.15 ± 0.08*

At 10min after [^{3}H]Trp (0.39kBq/kg B.W.) was injected to CCl_4-treated and control rats, radioactivity derived from the injected [^{3}H]Trp was determined in the liver and serum as described under Materials and methods.
[1]Each value is a mean ± SD ($n = 5$); *$p < 0.01$; **$p < 0.001$ (vs. control).

content of total hydroxyproline in the liver of the CCl_4-treated group was 7.5-fold more than that in the control group. The content of liver protein in the CCl_4-treated group was 1.6-fold more than that in the control group. The liver of CCl_4-treated group contained 2.5-fold more free Trp (non-protein Trp) than that of the control group. The total acitivity of liver TDO in rats with 12-week intermittent CCl_4 injection was 57% of that in control rats without CCl_4 treatment (Table 5). There was no significant difference in liver holo-TDO activity between the CCl_4-treated and control groups, although the activity tended to decrease in the CCl_4-treated group. The activity of apo-TDO in the liver of the CCl_4-treated group was 42% of that in the control group (Table 5).

When the transport of Trp into the liver of rats with and without 12 weeks of intermittent CCl_4 injection was examined in the pulse experiment using [^{3}H]Trp, the liver of the CCl_4-treated group had 2.1-fold higher radioactivity than that of the control group without CCl_4 treatment at 10min after [^{3}H]Trp injection (Table 6). Radioactivity in the serum of the CCl_4-treated group was 82% of that of the control group (Table 6).

Discussion

In the present study, rats with intermittent CCl_4 injection over a 12 week period showed an apparent liver injury, judging from the levels of

transminases, total protein, and albumin in the serum. In addition, liver cirrhosis occurred in the CCl_4-treated rats, judging from the level of total hydroxylproline in the liver. Sidransky et al. (1988) have shown morphologically that liver cirrhosis appears in rats with a similar long-term intermittent CCl_4 intoxication.

In the serum of rats with CCl_4-induced liver cirrhosis, total Trp levels were found to drop with a decrease in albumin-bound Trp levels as well as with an increase in free Trp levels, resulting in a reduction in the ratio of albumin-bound Trp to total Trp. Thus, an apparent increase in free serum Trp levels occurred in the cirrhotic rats. This result is well consistent with the results reported in patients with liver cirrhosis (Cangiano et al., 1976; Yoshida and Hirayama, 1980; Zoli et al., 1981; Hijita et al., 1981; Rocchi et al., 1986). The binding of Trp to albumin is well known to be affected not only by the concentration of albumin itself but also by long-chain fatty acids (Curzon et al., 1973; Curzon et al., 1974; Brodersen et al., 1989; Sasaki et al., 1993). In the cirrhotic rats, serum albumin levels decreased, while serum FFA levels did not change. In addition, there was no change in the ratio of albumin-bound Trp to albumin in the serum of the cirrhotic rats, indicating that the binding affinity of Trp to albumin does not change under cirrhotic conditions. Zoli et al. (1981) have shown that in cirrhotic patients, the decreased plasma albumin levels may play a causative role in the high plasma free Trp levels. Accordingly, it is suggested that the increase of free serum Trp levels found in rats with CCl_4-induced liver cirrhosis is mainly due to the reduction of serum albumin levels.

In the liver of rats with CCl_4-induced liver cirrhosis, there were increases in the contents of protein and free Trp, i.e., non-protein Trp and a marked decrease in the activity of TDO. The increase of liver protein content may be due to the maintenance of protein synthesis and the impaired secretion of proteins such as albumin. It has been shown that in rats with CCl_4-induced liver cirrhosis, synthesis of proteins such as albumin is normal or rather enhanced (Huberman and Sorberon, 1970; Kershenobich and Rojkind, 1973; Sidransky et al., 1988). It has also been reported that in cirrhotic patients, albumin synthesis is normal or elevated despite the reduction of plasma albumin levels (Hasch et al., 1967; Rothschild et al., 1969b). In the present study, albumin levels decreased in the serum of rats with CCl_4-induced liver cirrhosis. Trp is known to be the limiting amino acid for albumin synthesis in the liver under some circumstances (Rothschild et al., 1969a) and to stimulate protein synthesis in the liver of mice and rats (Sidransky, 1985). From these findings, it seems likely that the increase of the content of free Trp in the liver of rats with CCl_4-induced liver cirrhosis contributes to the maintenance of synthesis of proteins such as albumin in the tissue. It is known that under physiological conditions, a large part of Trp present in the plasma is taken up into the liver and then metabolized to acetyl CoA and NAD via the kynurenine pathway in which TDO takes part (Bender, 1982). TDO is not only the first enzyme in liver Trp metabolism but also the rate-limiting enzyme in the metabolism (Bender, 1982). From these findings, it seems that the reduction of liver TDO activity found in the cirrhotic rats contributes to an

enhancement of the utilization of free Trp taken up into the liver for synthesis of proteins such as albumin in the tissue. As to the reduction of liver TDO activity in the cirrhotic rats, this reduction was mainly dependent on the decrease of apo-TDO rather than holo-TDO. This finding suggests that under cirrhotic conditions, the synthesis of TDO itself rather than the binding of the enzyme to heme, a prosthetic group, could be impaired. It has been shown that in rats with acute liver injury induced by a single CCl_4 treatment, the synthesis of liver TDO is impaired at the translational, rather than the transcriptional step (Sato and Maruyama, 1972). Accordingly, it can be supposed that in the liver of rats with chronic CCl_4 liver injury, the activity of TDO is reduced through the impairment of its synthesis which may occur by a mechanism similar to the mechanism shown in acute CCl_4 liver injury.

From the matters described above, one can think the possibility that under cirrhotic conditions, increased free serum Trp is easily taken up into the liver and is able to be utilized for protein synthesis in the tissue, resulting in the reduction of total serum Trp concentration. Hence, we examined the transport of serum Trp into the liver of rats with liver cirrhosis induced by 12-week intermittent CCl_4 treatment in the pulse experiment using tritium-labled Trp. At 10 min after injection of [^{3}H]Trp into the portal vein of the CCl_4-treated and control rats, radioactivity derived from the injected [^{3}H]Trp in the liver of the CCl_4-treated rats was higher than that of the control rats, while the radioactivity in the serum of the former rats was lower than that of the latter rats. These results indicate that under cirrhotic conditions, serum Trp is easily taken up into the liver, resulting in the reduction of serum Trp levels and suggest the possibility that under cirrhotic conditions, synthesis of proteins such as albumin in the cirrhotic liver is maintained through such an easy transport of serum Trp into the tissue. However, it is required further investigation how the hepatic uptake of serum Trp is enhanced under cirrhotic conditions.

In conclusion, the present results indicate that although free Trp levels increase with decreases in total Trp, albumin-bound Trp, and albumin levels in the serum of rats with liver cirrhosis induced by long-term intermittent CCl_4 treatment, the increased Trp is easily taken up into the cirrhotic liver, and suggest that the Trp taken up into the cirrhotic liver could be utilized for the maintenance of synthesis of proteins such as albumin in the tissue through the reduction of Trp metabolism due to reduced TDO activity in the tissue. However, it is unclear at present why in the liver of rats with chronic CCl_4 liver injury, total TDO levels decreased despite the occurrence of enhanced albumin synthesis.

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