Glutathione S-Transferases and Chloroform Toxicity in Streptozotocin-Induced Diabetic Rats

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Abstract—The glutathione S-transferase activity in liver and kidney cytosol was significantly decreased in short term diabetes induced with streptozotocin, whereas no decrease in the transferase was observed in phenobarbital-treated diabetic rats. Toxicity of chloroform was potentiated in streptozotocin- or phenobarbital-treated rats. The decrease in liver cytosolic and microsomal glutathione S-transferase activity was observed in long term diabetic rats, and only microsomal transferase activity was restored by insulin treatment. There was no release of glutathione S-transferases into the serum in the diabetic rats, and the transferases were not inhibited by streptozotocin in vitro. These results showed that glutathione S-transferase activity decreased during diabetes, and this decrease may contribute to altering drug metabolism and toxicity in diabetes.

Glutathione S-transferases (EC 2.5.1.18) catalyze the reaction of glutathione conjugation with many xenobiotics and their reactive metabolites formed via the cytochrome P-450 monoxygenase system (1, 2). Evidence showed that drug metabolism and toxicity are altered in chemically-induced diabetes (3–7); however, there have been very few investigations of glutathione S-transferases in diabetic animals. It has been reported that hepatic glutathione S-transferases are altered by hepatotoxic chemicals and released into the serum (8, 9). In the present study, the effect of diabetes on glutathione S-transferase and chloroform toxicity was investigated.

Materials and Methods

Animal treatment for short term diabetes: Male Sprague-Dawley rats (Clea Japan, Inc., Tokyo), weighing 165–175 g, were used. The animals were starved for 16 hr prior to receiving 80 mg/kg streptozotocin in distilled water intraperitoneally (i.p.). Controls received an equivalent volume of water (0.3 ml/100 g). The rats were kept in a stainless steel wire cage with a 12 hr light-dark cycle and given a commercial laboratory chow (Clea, Japan, Inc., Tokyo) and water ad libitum. Seven days after the streptozotocin treatment, the animals were given 0.2 ml/kg chloroform, i.p., in a 30% olive oil solution. In the case of phenobarbital treatment, streptozotocin-diabetic and control rats were given phenobarbital sodium at 75 mg/kg, i.p., a day for 2 days, and chloroform was administered 18 hr after the last injection of phenobarbital. The rats were anesthetized with pentobarbital at 30 mg/kg, i.p., 5 hr after chloroform treatment. Immediately after the abdomen was opened by a midline incision, blood was collected from the inferior vena cava; and the liver and kidney were removed, weighed and perfused with 1.15% potassium chloride solution. The perfused liver and kidney were separately homogenized with a Teflon-glass homogenizer in 2 volumes of the same solution. The homogenate was centrifuged at 9,000×g for 30
min, and the supernatant fraction was further centrifuged at 105,000×g for 60 min. The supernatant thus obtained was used as the cytosol fraction, and the pellets from the livers were washed with 1.15% potassium chloride solution at 105,000×g for 60 min and then used as microsomes after resuspension in the same solution.

Animal treatment for long term (chronic) diabetes: Streptozotocin (100 mg/kg, i.p.) was administered to Sprague-Dawley rats under the same conditions as described for the short term diabetes, and the rats were killed 6 weeks later by decapitation. In the insulin treated group, rats were given insulin (4 units/animal/day, s.c.) from 3 days after streptozotocin injection until the day before killing. Liver cytosol and microsomes were prepared by the same method as described for the short term diabetic rats, except for twice washing of microsomes.

Enzyme assay: Glutathione S-transferase activity in the liver, kidney and serum was measured under the same conditions according to the method of Habig et al. (10), and activation of the microsomal enzyme by N-ethylmaleimide was done by the method of Morgenstern et al. (11). Aniline hydroxylase activity in liver microsomes was assayed under the same conditions as previously described (12). The serum glucose level and glutamate-pyruvate transaminase (GPT) activity were measured by using kits from Sinotest Laboratory, Ltd., Kanagawa, and Eiken Kagaku Ltd., Tokyo, respectively. In the in vitro experiments, liver cytosol and microsomes prepared from nontreated rats were incubated with 10 mM streptozotocin in 0.1 M potassium phosphate buffer (pH 7.4) in the presence of a NADPH generating system as described previously (12) at 37°C for 20 min, and then glutathione S-transferase activity was measured. Protein concentration in the cytosol and microsomes was determined by the method of Lowry et al. (13).

Statistical analysis: Data are expressed as the mean±S.D. Significance of the difference was calculated by Student's t-test, and P values<0.05 were taken as significant.

Chemicals: Streptozotocin and reduced glutathione were purchased from the Sigma Chemical Co., St. Louis, MO. 1-Chloro-2,4-dinitrobenzene (CDNB) and phenobarbital sodium were obtained from Wako Pure Chemical Industries, Ltd., Tokyo. Pentobarbital sodium was from Abbott Laboratories, North Chicago, IL. Lente insulin was purchased from Novo Industry, Denmark. 1,2-Dichloro-4-nitrobenzene (DCNB) and 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP) were obtained from Eastman Kodak, Co., Rochester, NY. Aniline was purified by distillation. All other chemicals were of analytical reagent grade.

Results

Tables 1 and 2 represent the change in body and tissue weights and the serum parameters.

Table 1. Body and tissue weights in short term diabetic rats

| Treatment | Body (g) | Liver (g/100 g b.w.) | Kidney (g/100 g b.w.) |
|-----------|---------|---------------------|---------------------|
| Nontreated |         |                     |                     |
| Control   | 264.3±9.2 | 5.00±0.51          | 0.79±0.07          |
| CHCl₃     | 255.9±13.5 | 4.60±0.13*         | 0.84±0.14          |
| STZ       | 214.3±26.3* | 4.88±0.19         | 1.09±0.09*         |
| STZ+CHCl₃ | 231.8±8.7* | 4.46±0.16*         | 1.00±0.08*         |
| PB treated |        |                     |                     |
| Control   | 258.2±15.2 | 4.97±0.34          | 0.72±0.03          |
| CHCl₃     | 270.0±11.7 | 4.67±0.34          | 0.73±0.06          |
| STZ       | 232.0±22.9 | 5.40±0.18*         | 0.96±0.06*         |
| STZ+CHCl₃ | 228.0±11.5* | 5.45±0.43*        | 1.04±0.19*         |

Streptozotocin (STZ, 80 mg/kg, i.p.), chloroform (CHCl₃, 0.2 ml/kg, i.p.) and phenobarbital (PB, 75 mg/kg, i.p.) were given to rats. Values represent the mean±S.D. from 4 to 11 rats. Analysis of variance: *P<0.05, control vs. treated; #P<0.05, CHCl₃ vs. STZ+CHCl₃.
Table 2. Serum parameters in short term diabetic rats

| Treatment          | Glucose (mg/dl) | SGPT (Karmen unit) | SGST (µmol/ml) |
|--------------------|-----------------|--------------------|----------------|
| Nontreated         |                 |                    |                |
| Control            | 189.0±20.3      | 16.5±1.8           | 0.08±0.01      |
| CHCl₃              | 189.5±17.4      | 32.7±15.4*         | 0.10±0.03      |
| STZ                | 440.9±69.3*     | 24.8±6.3*          | 0.08±0.01      |
| STZ+CHCl₃          | 397.2±34.9*     | 60.9±30.2*         | 0.09±0.02      |
| PB treated         |                 |                    |                |
| Control            | 153.6±10.1      | 17.6±2.8           | 0.06±0.01      |
| CHCl₃              | 152.2±7.7       | 798.3±555.5        | 3.00±2.12      |
| STZ                | 434.6±58.1*     | 27.5±5.7*          | 0.07±0.01      |
| STZ+CHCl₃          | 288.3±75.5*     | 5342.4±5019.7      | 8.83±8.53      |

Rats were given chemicals as described in Table 1 and glucose, glutamate-pyruvate transaminase (SGPT) and glutathione S-transferase (SGST) in serum were measured as described in Materials and Methods. Values represent the mean±S.D. from 4 to 11 rats. Analysis of variance: *P<0.05, control vs. treated; #P<0.05, CHCl₃ vs. STZ+CHCl₃; †P<0.05, STZ vs. STZ+CHCl₃.

in short term diabetic rats. A significant decrease in body weight (81% of the control) and an increase in serum glucose levels (440.9±69.3 mg/dl) were observed in streptozotocin-treated rats. These results showed that rats treated with streptozotocin were in a diabetic state. In the phenobarbital treated groups, the serum glucose level (434.6±58.1 mg/dl) in the diabetic rats was the same as that in the nontreated rats, but it was significantly decreased (288.3±75.5 mg/dl) by chloroform treatment. Liver weight per 100 g body weight was decreased by chloroform treatment. Kidney weight was significantly increased by streptozotocin treatment, and the kidney seemed to become hydropnephrotic. A phenomenon of alteration of tissue weights similar to that shown in nontreated rats was observed in the phenobarbital treated groups except that an increased liver weight in the diabetic animals was noted.

Serum GPT activity in nontreated rats was increased to 1.9-times that of the control after chloroform treatment and 3.6-times in the combined streptozotocin and chloroform groups. Serum glutathione S-transferase activity was not increased by chloroform treatment. In phenobarbital-treated groups, GPT activity was markedly increased to 45-times the control level by chloroform alone and to 303-times the control level by the combination of chloroform and streptozotocin. Serum glutathione S-transferase activity after chloroform treatment was 46-times the control level in nondiabetic and 138-times the control level in the diabetic rats. However, these marked elevations of both serum GPT and glutathione S-transferase activities were not statistically significant because of the broad deviation at high concentrations.

As shown in Table 3, liver cytosolic glutathione S-transferase activity in nontreated groups was significantly decreased to 79% of the control by treatment with chloroform, to 75% with streptozotocin and to 67% by a combination of both chemicals. Kidney cytosolic glutathione S-transferase activity was significantly decreased only in streptozotocin-diabetes. In phenobarbital treated rats, the diabetic state alone caused no significant decrease in glutathione S-transferase activity in both the liver and kidney. Aniline hydroxylation was increased slightly (116% of the control) in the diabetic rats of the nontreated group, but the hydroxylase activity reached the level of the control after they were given chloroform. In the phenobarbital treated rats, the hydroxylation was not altered in the diabetic state. Chloroform treatment caused a significant decrease in aniline hydroxylation in both nontreated and phenobarbital treated groups.

In chronic diabetic rats, body weight was decreased to 44% of the control and recovered to 93% in insulin-treated rats. Glutathione S-transferase activity was not detected in the
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Table 3. Glutathione S-transferase and aniline hydroxylase activities in short term diabetic rats

| Treatment          | GST for CDNB (µmol/mg/min) | Aniline hydroxylase (p-Aminophenol nmol/20 min) |
|--------------------|----------------------------|-----------------------------------------------|
|                    | Liver cytosol              | Kidney cytosol                               |
| Nontreated         |                            |                                               |
| Control            | 2.604±0.47                 | 0.527±0.10                                   | 22.79±3.72                                  |
| CHCl₃              | 2.063±0.36*                | 0.476±0.08                                   | 17.56±1.60*                                 |
| STZ                | 1.970±0.45*                | 0.422±0.02*                                  | 26.54±1.44                                  |
| STZ+CHCl₃          | 1.748±0.20*                | 0.471±0.03                                   | 21.85±3.90*                                 |
| PB treated         |                            |                                               |
| Control            | 2.848±0.28                 | 0.746±0.16                                   | 29.57±2.04                                  |
| CHCl₃              | 1.525±0.06*                | 0.646±0.11                                   | 19.21±1.20*                                 |
| STZ                | 2.544±0.25                 | 0.693±0.11                                   | 31.46±9.57                                  |
| STZ+CHCl₃          | 1.416±0.28*                | 0.487±0.16                                   | 17.72±8.67*                                 |

Values represent the mean±S.D. from 4 to 11 rats. Analysis of variance: *P<0.05, control vs. treated; #P<0.05, CHCl₃ vs. STZ+CHCl₃.

Table 4. Liver cytosolic glutathione S-transferase activity in chronic diabetic rats

| Treatment         | CDNB (µmol/mg/min) | DCNB (nmol/mg/min) | EPNP (nmol/mg/min) |
|-------------------|--------------------|--------------------|--------------------|
|                   |                    |                    |                    |
| Control           | 1.98±0.35 (100±17) | 92.32±28.61 (100±29) | 59.25±20.63 (100±36) |
| STZ               | 1.34±0.59* (67±30) | 53.87±15.83* (66±16) | 58.90±14.19 (68±29) |
| STZ+Insulin       | 1.35±0.29* (68±15) | 64.82±15.12 (67±16) | 55.76±20.01 (68±33) |

Values represent the mean±S.D. from 4 to 8 rats. Analysis of variance: *P<0.05 control vs. treated.

serum of diabetic rats. Serum glucose levels in the chronic diabetic and insulin-treated rats were 582.2±31.4 and 145.5±6.3 mg/dl, respectively. These results indicate that the diabetic state caused by streptozotocin was normalized by insulin treatment. Table 4 shows liver cytosolic glutathione S-transferase activity in chronic diabetes. The cytosolic glutathione S-transferase activity for CDNB and for DCNB was significantly decreased to 67% and to 56% of the control, respectively, but the transferase activity for EPNP was not altered. The decrease in glutathione S-transferase activity for CDNB and DCNB was not restored by insulin treatment.

Liver microsomal glutathione S-transferase activity in chronic diabetic rats, as shown in Table 5, was significantly decreased to 75% of the control; in contrast to the cytosolic transferase, the decrease was restored to the control level by insulin treatment. The activation ability of N-ethylmaleimide (NEM) to the microsomal glutathione S-transferase (as shown in percent of increase) was not altered in either the chronic diabetic or insulin-treated groups, and the restoration of the activity by insulin was also observed for the NEM-activated enzyme. Neither cytosolic nor microsomal glutathione S-transferase was altered by incubation with streptozotocin in the presence of the NADPH-generating system (Table 6).

Discussion

How a short term and long term (chronic) diabetic state in rats affects glutathione S-transferase was investigated in combination with chloroform treatment. Hepatic glutathio-
Table 5. Liver microsomal glutathione S-transferase activity in chronic diabetic rats

| Treatment   | Microsomal GST for CDNB (μmol/mg/min) | unactivated | NEM-activated | activation (%) |
|-------------|--------------------------------------|------------|---------------|---------------|
| Control     |                                      | 0.031±0.007| 0.680±0.072   | 747±79        |
|             |                                      | (100±8)    | (100±10)      |               |
| STZ         |                                      | 0.069±0.01*| 0.515±0.084*  | 817±149       |
|             |                                      | (75±11)    | (75±13)       |               |
| STZ+Insulin |                                      | 0.089±0.009| 0.716±0.099   | 832±115       |
|             |                                      | (98±10)    | (105±14)      |               |

Values represent the mean±S.D. from 4 to 8 rats. Analysis of variance: *P<0.05, control vs. treated; #P<0.05, STZ vs. STZ+Insulin.

Table 6. Effect of STZ on liver cytosolic and microsomal glutathione S-transferase activity in vitro

| Treatment    | GST activity for CDNB (μmol/mg/min) |
|--------------|-------------------------------------|
|              | Cytosol | Microsomes | Cytosol+Microsomes |
| Control      | 1.118±0.08 | 0.167±0.02 | 0.137±0.01 |
| STZ (10 mM)  | 1.141±0.04 | 0.174±0.01 | 0.145±0.01 |

Values represent the mean±S.D. from 3 incubations at 37°C for 20 min.

one S-transferase activity was significantly decreased in the short term diabetic rats, and this decrease was additive when chloroform was administered. However, no decrease in hepatic glutathione S-transferase was observed in phenobarbital-treated diabetic rats (Table 3). This may be due to an induction of hepatic glutathione S-transferase because phenobarbital is known as a glutathione S-transferase inducer (15). Though kidney weight increased more than liver weight, and the kidneys seemed to become hydronephrotic in short term diabetic rats, the renal cytosolic glutathione S-transferases were not altered as much as the hepatic cytosolic S-transferase. Furthermore, we observed that heart cytosolic glutathione S-transferase was not affected in streptozotocin-induced diabetic rats (Y. Aniya et al., unpublished data). It is, therefore, suggested that the effect of a diabetic state on glutathione S-transferase varies according to the organ species.

Aniline hydroxylation was slightly increased in the diabetic groups and the same degree of increase in hydroxylation was reported (16). Chloroform treatment caused a significant decrease of both liver cytosolic glutathione S-transferase and microsomal aniline hydroxylase in either nontreated or phenobarbital treated rats. Since chloroform was converted to active metabolites via the cytochrome P-450 system (17), microsomal aniline hydroxylase might have been attacked suicidally by the metabolites. Potentiation of chemically-induced toxicity in the diabetic state has been previously reported (5, 7, 14), and the potentiation was mainly attributed to an alteration of drug-metabolizing enzymes. In the present study, the potentiation of chloroform toxicity, as judged from elevated GPT and bilirubin levels in serum, was also observed in diabetic and phenobarbital treated rats. Thus, an induction of the cytochrome P-450, as shown in the small increase of aniline hydroxylase, may contribute to potentiation of chloroform toxicity in the diabetic state. In addition, the decrease of the glutathione S-transferase activity may play a role in potentiation of chloroform toxicity.

In order to clarify the alteration of hepatic glutathione S-transferases, we studied long term (chronic) diabetes caused by a high dose of STZ (100 mg/kg, i.p.). As judged from the alteration of blood glucose level and body
weight, chronic diabetes is a severe diabetic condition compared to short term diabetes. Under these conditions, hepatic cytosolic glutathione S-transferase activity was decreased for CDNB and DCNB but not for EPNP. Thus, it is considered that among the several isoenzymes in the hepatic cytosol, glutathione S-transferase 3-3 or 5-5, which is known to have substrate specificity to EPNP (1), is not affected by diabetes. This may mean that the diabetic state has a different effect on each isoenzyme of hepatic glutathione S-transferase, as has been reported for cytochrome P-450 species (18-20). Furthermore, although the decrease of cytosolic glutathione S-transferase activity was not restored by insulin treatment, blood glucose level and body weight were normalized by insulin. Therefore, it is considered that hepatic cytosolic glutathione S-transferase is less sensitive to insulin than microsomal glutathione S-transferase. Concerning glutathione S-transferase in diabetic animals, an increase in mice (21) and a decrease in rats (22) were reported. Younes et al. (22) showed that rat liver cytosolic glutathione S-transferases in short term (4 days) diabetes were decreased and restored by insulin treatment. This difference may be, in part, due to when insulin treatment was started, because we initiated it on the third day after streptozotocin injection, but they started the treatment on the day of streptozotocin injection. A partial restoration by insulin treatment of other enzymes that had been altered in diabetic animals was reported (23).

In contrast to cytosolic glutathione S-transferase, the decreased microsomal glutathione S-transferase activity in chronic diabetes was restored by insulin treatment. This difference between cytosolic and microsomal glutathione S-transferases in insulin treatment may reflect a difference in the regulating mechanism of both enzymes in vivo. It has been reported that insulin receptors are modified by sulfhydryl agents (24), and the hormone action may be mediated by hydrogen peroxide (25). It is well-known that hepatic microsomal glutathione S-transferase is activated by sulfhydryl agents (11). Recent data show that hydrogen peroxide increases hepatic microsomal glutathione S-transferase activity (26). Thus it is assumed that the membrane-bound microsomal glutathione S-transferase may be affected by insulin.

It is of interest that a decrease in hepatic glutathione S-transferase activity continues for a long time as seen in chronic diabetes. Since no release of the glutathione S-transferase into the serum was observed during diabetes, a mechanism different from chemically-induced decrease as shown in chloroform treatment may be related to the decrease of the transferase in the diabetic state. In our in vitro experiments, neither cytosolic nor microsomal glutathione S-transferase was inhibited by the addition of streptozotocin (Table 6). This means that reactive metabolites from streptozotocin formed via the cytochrome P-450 system or streptozotocin itself do not contribute to a decrease in either cytosolic or microsomal glutathione S-transferase activity. Because streptozotocin causes an impairment of DNA for insulin synthesis by free radical formation (27), the same phenomenon may occur on the gene for glutathione S-transferase. Alternatively an inhibitory regulation on glutathione S-transferases may be caused by metabolic changes during diabetes. Further study is needed to clarify the regulating mechanism of glutathione S-transferases in the diabetic state.

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