Effects of 8-(2-Dimethylaminoethyl)-3-Oxo-4-Phenyl-1-Thia-4,8-Diazaspiro [4,5] Decane Dihydrochloride Monohydrate (Y-8845) on Carbon Tetrachloride-Induced Liver Injury

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Accepted January 9, 1986

Abstract—The effects of 8-(2-dimethylaminoethyl)-3-oxo-4-phenyl-1-thia-4,8-diazaspiro [4, 5] decane dihydrochloride monohydrate (Y-8845) on carbon tetrachloride (CCl₄)-induced liver injury were investigated in rats. CCl₄-induced attenuation of the plasma cyclic AMP (cAMP) response to glucagon stimulation was significantly prevented by pretreatment with Y-8845. Y-8845 also effectively suppressed the increases in the activities of serum transaminases as well as the decreases in microsomal glucose-6-phosphatase activity and microsomal cytochrome P-450 concentrations induced by CCl₄. In rats at 72 hr after CCl₄ administration, the plasma cAMP response to glucagon, microsomal glucose-6-phosphatase activity and P-450 concentration were all below the control level. Y-8845 treatment after CCl₄ administration rectified these reductions to nearly normal levels. Furthermore, Y-8845 stimulated DNA synthesis during liver regeneration after CCl₄ intoxication. These results demonstrate that Y-8845 has a protective effect against CCl₄-induced injury in the liver and a stimulating effect on the recovery of the damaged liver.

A new compound, 8-(2-dimethylaminoethyl)-3-oxo-4-phenyl-1-thia-4,8-diazaspiro [4, 5] decane dihydrochloride monohydrate (Y-8845; the chemical structure of which is shown in Fig. 1) has been reported to have a hepatoprotective effect on various kinds of experimental injuries (1). Pretreatment with Y-8845 is said to reduce the increases in serum transaminase activities induced by carbon tetrachloride (CCl₄), thioacetamide, D-galactosamine, α-naphthylisothiocyanate and endotoxin (1). Furthermore, it has been found that Y-8845 demonstrates anti-fibrotic action on egg yolk-induced chronic fibrosis (1). However, the effects of Y-8845 on the recovery of liver after intoxication are as yet less well defined. Recently, it has been reported that the cyclic AMP (cAMP) response to glucagon and DNA synthesis in liver may be a valuable indicator for the recovery of hepatic injury (2). The aim of the present study is to provide further information as to the effects of Y-8845 on protection against and the acceleration of recovery from hepatic injury in CCl₄-intoxicated rats.

Materials and Methods

Treatment of animals: Male Wistar rats (250–320 g) fed standard laboratory chow (Oriental-MF) ad lib. were used for the experiments.
CCl₄ (1 ml/kg) in 50% olive oil solution was administered to rats by a stomach tube. Normal control rats received olive oil alone.
Y-8845 was dissolved in saline and administered to three groups of rats intra-peritoneally: 1) at 30 min before CCl₄ administration, 2) at 24 and 36 hr after CCl₄ administration, and 3) at 24, 36, 48 and 60 hr after CCl₄ administration.

Preparation of liver microsomes: At 24 or 72 hr after CCl₄ administration, livers were removed after in situ perfusion with saline, and a 20% liver homogenate was prepared in 0.15 M KCl solution using a Polytron homogenizer. The homogenate was centrifuged at 12,000 x g for 20 min. The resultant supernatant was centrifuged at 105,000 x g for 60 min, and the microsomal pellet was washed once with 0.15 M KCl solution. The washed microsomes were finally suspended in 0.1 M phosphate buffer, pH 7.0, for the measurement of cytochrome P-450 content. Protein was determined by the method of Lowry et al. (3).

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Determination of plasma cAMP level: Under pentobarbital anesthesia, blood specimens (0.05 ml) were taken from the tail vein at intervals of 10 min before and after the injection of glucagon (100 \( \mu \)g/kg, s.c.) and were immediately mixed with 0.2 ml of saline containing 10 mM EDTA. After centrifugation, the supernatant (0.1 ml) was analyzed for cyclic AMP by radioimmunoassay (4) using a commercial assay kit (Yamasa, Japan).

Determination of hepatic DNA synthesis: DNA synthesis in liver was determined as described previously (2). In brief, rats were injected intraperitoneally with 5 \( \mu \)Ci (methyl-\(^3\)H)thymidine per 100 g body weight and killed 1 hr later. DNA was extracted from the liver homogenate as described by Munro and Fleck (5), and the amount of radioactivity incorporated into DNA was measured by counting on a Beckman LS 9000 scintillation spectrometer. DNA was measured by the method of Martin et al. (6) using calf thymus DNA standards. \(^3\)H-Thymidine incorporated into liver DNA was expressed as cpm/mg DNA.

Determination of enzyme activities: G6Pase activity in hepatic microsomes was measured as described by Swanson (7) and liberated inorganic phosphate was measured by the method of Ames (8). Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities in serum were determined using a commercial assay kit (Iatron, Tokyo). Cytochrome P-450 content in microsomal preparations was measured according to the procedure described by Omura and Sato (9).

Results

Protective effects of Y-8845 against CCl₄-induced liver injury: The protective action of Y-8845 against CCl₄-induced liver injury was observed in the changes in serum GOT and GPT activities. As shown in Fig. 2, a marked elevation of serum GOT and GPT activities in rats resulted from CCl₄ intoxication 24 hr after the toxin administration. The pretreatment of rats with Y-8845 (30 or 100 mg/kg) significantly inhibited the development of CCl₄-induced elevations of serum transaminase activities. Although both liver microsomal G6Pase activity and cytochrome P-450 content were markedly decreased at 24 hr after CCl₄ administration, Y-8845 (100 mg/kg) given 30 min before CCl₄ significantly suppressed the CCl₄-induced reductions of G6Pase activity and P-450 content (Fig. 3). As a parameter to observe the state of hepatic plasma membrane functions (10), the changes of plasma cAMP levels after glucagon injection were investigated in CCl₄-intoxicated rats with or without Y-8845 pretreatment. These results are shown in Table 1. When glucagon (100 \( \mu \)g/kg, s.c.) was injected into normal rats, plasma cAMP strikingly increased and reached a peak (4.2 times higher than the basal plasma level) at 20 min after the injection. This glucagon-induced response in the plasma cAMP level was almost abolished
in rats at 24 hr after CCl₄ administration. On the other hand, pretreatment with Y-8845 (100 mg/kg, i.p.) 30 min before CCl₄ administration significantly diminished the CCl₄-induced attenuation in the plasma cAMP response to glucagon. This Y-8845 effect was observed at a dosage as low as 10 mg/kg. A single injection of Y-8845 in normal rats had no substantial influence on the glucagon-induced increase in plasma cAMP.

Effects of Y-8845 on the recovery from impairment of liver: Two aspects of the
Table 1. Effect of Y-8845 on the plasma cyclic AMP response to glucagon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Plasma cyclic AMP (pmol/ml)</th>
<th>Time after injection of glucagon (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive oil + saline</td>
<td>12</td>
<td>59.4±5.3</td>
<td>133.9±23.8</td>
<td>251.4±63.7</td>
<td>110.3±29.9</td>
<td>79.9±10.1</td>
<td></td>
<td></td>
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<tr>
<td>+Y-8845 (10 mg/kg)</td>
<td>8</td>
<td>54.8±5.0</td>
<td>147.2±26.3</td>
<td>264.0±65.3</td>
<td>126.1±27.6</td>
<td>83.6±19.7</td>
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<td></td>
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<tr>
<td>+Y-8845 (30 mg/kg)</td>
<td>8</td>
<td>49.8±5.2</td>
<td>122.1±17.5</td>
<td>206.7±54.8</td>
<td>113.2±24.4</td>
<td>84.8±15.4</td>
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<td></td>
</tr>
<tr>
<td>+Y-8845 (100 mg/kg)</td>
<td>8</td>
<td>50.4±4.4</td>
<td>134.5±50.9</td>
<td>267.7±67.1</td>
<td>118.5±20.1</td>
<td>84.1±13.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCl₄ + saline</td>
<td>10</td>
<td>44.6±5.9</td>
<td>48.8±8.7</td>
<td>61.0±12.0</td>
<td>60.3±10.5</td>
<td>46.5±8.1</td>
<td></td>
<td></td>
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<tr>
<td>+Y-8845 (10 mg/kg)</td>
<td>8</td>
<td>46.8±4.6</td>
<td>60.4±7.7</td>
<td>100.3±8.9*</td>
<td>56.4±7.1</td>
<td>60.0±4.8</td>
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<td></td>
</tr>
<tr>
<td>+Y-8845 (30 mg/kg)</td>
<td>8</td>
<td>47.3±6.5</td>
<td>74.4±12.0</td>
<td>124.6±18.0</td>
<td>96.9±9.5*</td>
<td>74.3±11.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Y-8845 (100 mg/kg)</td>
<td>8</td>
<td>53.5±5.1</td>
<td>118.9±30.6*</td>
<td>174.8±20.1</td>
<td>72.1±11.7</td>
<td>68.1±10.5</td>
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Y-8845 was injected intraperitoneally 30 min before the administration of CCl₄ (1 ml/kg, p.o.). Twenty-four hr after the CCl₄ administration, glucagon (100 μg/kg, s.c.) was injected into rats at time 0. Each value is given as the mean±S.E. *P<0.05, **P<0.01, compared with CCl₄+saline.

Table 2. Effect of Y-8845 on the plasma cyclic AMP response to glucagon in rats at 72 hr after CCl₄ administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Plasma cyclic AMP (pmol/ml)</th>
<th>Time after injection of glucagon (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive oil + saline</td>
<td>6</td>
<td>31.8±3.5</td>
<td>131.7±31.5</td>
<td>241.3±44.0</td>
<td>175.7±52.5</td>
<td>89.5±18.2</td>
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<tr>
<td>+Y-8845 (2 times)</td>
<td>5</td>
<td>36.6±9.3</td>
<td>139.5±34.9</td>
<td>285.6±19.2</td>
<td>164.3±18.2</td>
<td>78.7±15.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Y-8845 (4 times)</td>
<td>5</td>
<td>49.5±4.7</td>
<td>201.6±58.6</td>
<td>322.1±74.2</td>
<td>144.1±28.2</td>
<td>115.2±18.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCl₄ + saline</td>
<td>5</td>
<td>34.7±5.3</td>
<td>114.2±32.3</td>
<td>160.1±25.9</td>
<td>102.9±27.0</td>
<td>57.9±11.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Y-8845 (2 times)</td>
<td>6</td>
<td>38.3±4.1</td>
<td>136.2±26.9</td>
<td>221.5±48.9</td>
<td>135.9±22.4</td>
<td>93.2±19.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Y-8845 (4 times)</td>
<td>6</td>
<td>29.9±2.9</td>
<td>178.4±43.9</td>
<td>243.0±48.3</td>
<td>127.8±42.1</td>
<td>87.0±23.9</td>
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</tr>
</tbody>
</table>

Y-8845 (100 mg/kg) was injected intraperitoneally 2 times at 24 and 36 hr after CCl₄ administration or 4 times at 24, 36, 48 and 60 hr. Seventy-two hr after CCl₄ administration, glucagon (100 μg/kg, s.c.) was injected into rats at time 0. Each value is given as the mean±S.E.
recovery of hepatic impairment in CCl₄-intoxicated rats were examined: responsiveness to glucagon and increase in DNA synthesis, with or without Y-8845 treatments.

In rats at 72 hr after CCl₄ intoxication, the plasma cAMP response to glucagon remained lower than in control rats. As shown in Table 2, treatment with Y-8845 (100 mg/kg) at 24 and 36 hr or at 24, 36, 48 and 60 hr after CCl₄ administration manifested a pronounced tendency to improve the toxin-induced impairment in cAMP response to glucagon.

The capacity of recovery from the CCl₄ injury was examined by measurement of DNA synthesis. Figure 4 shows the effect of Y-8845 on hepatic DNA synthesis in CCl₄-treated rats. The value of basal DNA synthesis (without the drug) in control rats was 1949 ±147 cpm/mg DNA. Though the DNA synthesis significantly increased (3622±342 cpm/mg DNA) 48 hr after CCl₄ administration, indicating liver regeneration after the intoxication (11), administration of Y-8845 (30 or 100 mg/kg) to rats at 24 and 36 hr after the intoxication caused an enhancement of DNA synthesis. However, even a large dose (100 mg/kg) of Y-8845 did not affect hepatic DNA synthesis in control animals. Also there was no appreciable difference in hepatic DNA synthesis between the Y-8845 (100 mg/kg)-treated and saline-treated animal groups at one week after CCl₄ intoxication.

Discussion

In the present study, a single dose of Y-8845 (10–100 mg/kg) given 30 min before CCl₄ significantly diminished the CCl₄-induced attenuation of the plasma cAMP response to glucagon (Table 1). Glucagon, regulating hepatic glycogenolysis and gluconeogenesis through an increase in cellular cAMP (12, 13), induces an elevation of the plasma cAMP by an increased efflux of the nucleotide from the liver (14). In line with our previous report (2), the response was almost abolished at 24 hr after the toxin (Table 1). Coincidently, the binding of glucagon to hepatic plasma membrane was diminished from 24 to 48 hr after the intoxication (15). It may be considered that Y-8845 is capable of protection against CCl₄-induced damage on the hepatic plasma membrane. This supposition is supported by the facts that the CCl₄-induced transaminase
leakage from hepatocytes to plasma, the decrease in cytochrome P-450 and the reduction in G6Pase activity were attenuated by Y-8845 administration prior to the toxin (Figs. 2 and 3). The mechanism of the protective action of Y-8845 is unclear at present. It is reported that the destruction of G6Pase of cytochrome P-450 by CC14 in vivo correlated with the enhancement of lipid peroxidation (16). Therefore, the protective effect of Y-8845 against CC14 hepatotoxicity observed in this study might be partly ascribable to anti-peroxidation properties.

An improvement of the response to glucagon was found with repeated administration of Y-8845 to the intoxicated rats, but it was not statistically significant when compared with non-treated intoxicated animals. The injury of hepatocytes occurs synchronously in the early stages of CC14 intoxication (12–24 hr), but after the acute alteration of hepatocytes induced by a single dose of the toxin, cells in various stages of recovery from the damage may appear in the whole liver (17). The glucagon-responses of these hepatocytes in various stages of recovery revealed no significant statistical differences between CC14-treated rats and those treated with olive oil, or between the intoxicated rats treated with Y-8845 and those given no drug (Table 2).

In the present study, change of hepatic DNA synthesis was investigated as a hepatic response of repair from CC14-injury. Y-8845 given at 24 hr after CC14 potentiated hepatic DNA synthesis at 48 hr in the intoxicated rats (Fig. 4), but this potentiating effect was not dose-dependent in the used dose range, suggesting the maximal response to the drug. From our previous studies (2, 18), it seems likely that hepatic DNA synthesis of rats given CC14 (1 ml/kg) reaches the maximal value at 48 hr and returns to roughly the normal level at 72 hr and that DNA synthesis in CC14-injured liver is more sensitive to stimulants (e.g., hormones and neurotransmitters) for a period of 24 to 48 hr after the intoxication. The absence of the dose-dependency on potentiation of DNA synthesis by the drug may have resulted from this sensitized state of CC14-injured liver, and the dose-dependency may be obtained with smaller doses of Y-8845. This assumption is supported by the facts that the second dose

Fig. 5. Effects of Y-8845 on microsomal content of cytochrome P-450 (Cyt. P-450) (A) and glucose-6-phosphatase (G6Pase) activity (B) in rats at 72 hr after CC14 administration. Y-8845 at 100 mg/kg (Y(100)) was injected intraperitoneally 4 times at 24, 36, 48 and 60 hr after CC14 administration. Hepatic microsomes were prepared from rats at 72 hr after CC14 administration, and P-450 content and G6Pase activity were measured. Each bar is given as the mean±S.E. of 5–6 rats. §P<0.01, §§P<0.001, compared with olive oil+saline. *P<0.05, compared with CC14+saline.
of Y-8845 did not augment the effect of the first dose on DNA synthesis and that Y-8845 failed to increase the DNA synthesis in one week-passed rats after CCl₄ administration.

The accelerated DNA synthesis may promote a rapid recovery in cytochrome P-450 content and G6Pase activity, since these CCl₄-induced dysfunctions were prolonged until 72 hr after the CCl₄ intoxication.

The present study suggests that Y-8845 has both a protective action against CCl₄-induced injury and an action accelerating the recovery from CCl₄-induced damage.

Acknowledgment: We wish to thank to Dr. Katsuji Hoshi, Hokkaido Institute of Pharmacy, for his valuable advice.

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