Suppressive Effect of Tritoqualine on Lipid Peroxidation and Enzyme Leakage Induced by Carbon Tetrachloride in Rat Hepatocytes

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Abstract—Since tritoqualine (TRO) is effective in suppressing the increase of serum transaminases in acute hepatic injured rats induced by some hepatotoxins, protection of the hepatocyte membrane is suggested to be one of the pharmacological effects of TRO. In the present study, we investigated the effects of TRO on lipid peroxidation and enzyme leakage caused by carbon tetrachloride (CCl₄) exposure in isolated hepatocytes and the liver in vivo, compared with vitamin E. The results were as follows: 1) Hepatocytes isolated from TRO-administered rats showed less enzyme leakage than those from control rats after CCl₄ addition. 2) TRO displayed strong inhibition of lipid peroxidation in isolated hepatocytes. In comparison with vitamin E, TRO showed almost the same inhibitory action on lipid peroxidation, but a stronger suppression of enzyme leakage. 3) Vitamin E showed a weaker protection from increase of glutamic oxaloacetic transaminase than TRO, in spite of its stronger inhibition of lipid peroxidation in vivo. From these results, it is suggested that the membrane protecting action of TRO is partially derived from its suppression of lipid peroxidation, but “another action” may also play an important role in protecting the fragile membrane.

Tritoqualine (TRO), which is a derivative of isoquinoline phthalide (Fig. 1), has been reported to be effective on patients with chronic active hepatitis (1, 2). We previously reported that TRO administration preventively and therapeutically improved various parameters which represented abnormalities of hepatic function in chronically injured rats by carbon tetrachloride (CCl₄) (3, 4). We also revealed that TRO pretreatment markedly suppressed increase of serum transaminases induced by single administration of CCl₄, D-galactosamine or allyl alcohol in rats (5). From these experiments, it was suggested that TRO was a protective agent for the liver, and that the protection of hepatocyte membrane was one of the pharmacological effects of TRO.

CCl₄ is a hepatotoxin which causes membrane fragility, enzyme leakage and eventual necrosis in the liver. In addition, it is well-known that peroxidation of membrane lipids occurs in an early stage of CCl₄ metabolism and contributes to membrane fragility (6). In order to investigate the action of TRO on hepatocyte membrane, we studied its effect on lipid peroxidation in isolated hepatocytes

TRQ  (Tritoqualine)

Fig. 1. Chemical structure of tritoqualine (TRO).
and the liver in vivo by using CCl₄, compared with vitamin E, an endogenous antioxidant (7).

Materials and Methods
Reagents: TRQ was synthesized at Mitsubishi Chemical Ind., Ltd. Vitamin E (α-tocopherol) and phenobarbital sodium were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). BSA (bovine serum albumin, Fraction V), HEPES (N-2-hydroxyethylpiperazine-N’-2-ethane-sulphonic acid), EGTA (ethyleneglycol-bis-(β-amino-ethyl ether) N,N’-tetraacetic acid) and Tween 80 (polyoxyethylene sorbitan mono-oleate) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Collagenase (CLS IV) and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. (Freehold, U.S.A.). TBA (2-thiobarbituric acid) was obtained from E. Merck AG (Darmstadt, F.R.G.). Other reagents used were all analytical grade.

CCI₄-induced enzyme leakage from isolated rat hepatocytes: Male Wistar rats weighing about 150 g obtained from Japan Laboratory Animals, Inc. (Tokyo, Japan) were fed laboratory chow and tap water ad lib. TRQ was suspended in 0.2% Tween 80 solution and given to the rats (200 mg/kg, p.o.) daily for 7 days. The same amount of 0.2% Tween 80 solution was administered to the control rats instead of TRQ. Hepatocytes were isolated from a perfused liver by a modified Seglen’s procedure (8). Briefly, perfusion of the liver was started with 150 ml of a 10 mM HEPES-buffered Ca²⁺-free Hanks’ solution (pH 7.4) containing 0.5 mM EGTA. After 5 min, the liver was excised, and the perfusate was changed to 100 ml of a recirculating HEPES-buffered Hanks’ solution (pH 7.4) containing 5 mM CaCl₂, 0.05% collagenase and 0.005% soybean trypsin inhibitor instead of EGTA. The process continued for 15–20 min at 37°C with aeration of carbogen (95% O₂, 5% CO₂), until the liver began to disintegrate visibly. Cells were dispersed in a HEPES-buffered Hanks’ solution (pH 7.4), filtered through a nylon stocking, and sedimented by triple centrifugation for 1 min at 50×g. Finally, the hepatocytes were suspended at 5×10⁶ cells/ml in the same solution supplemented with 2% BSA. Two ml of the cell suspension was placed on 35 mm diameter culture dishes (Falcon Plastics, Oxnard, U.S.A.) and incubated in an incubator at 37°C. CCl₄ was diluted with ethanol (10%, v/v) and added to the dishes at a concentration of 2.6 or 5.2 mM (0.25 or 0.5%, v/v, respectively). As a marker of cell membrane damage, lactate dehydrogenase (LDH, EC 1.1.1.27) leaked out from the cells was determined by the method of Moldéus et al. (9) using the supernatant of centrifuged samples. Total LDH activity was measured after lysis of the cells by sonication. The results are expressed as a ratio of LDH release induced by CCl₄ to total LDH in the cells (=LDH leakage induced by CCl₄ diluted with ethanol–LDH leakage induced by ethanol)/(total activity–0 time background).

CCI₄-stimulated lipid peroxidation in isolated rat hepatocytes: Rats were pretreated with phenobarbital sodium (1 mg/ml of drinking water) for at least 7 days prior to use for cell preparation in order to induce liver microsomal cytochrome P-450 (10). Isolated hepatocytes were prepared from the rats (200–250 g) as mentioned above. The hepatocytes were suspended at 1×10⁶ cells/ml in the HEPES-buffered Hanks’ solution (pH 7.4) containing 1% BSA. The test compounds, TRQ and vitamin E, were dissolved in 0.1 N HCl and DMSO, respectively. Ten ml of the cells were added to 50 ml Erlenmeyer flasks containing 50 μl of the test compounds, and incubated at 37°C in a shaking water bath. After one hour of incubation, CCl₄ was added to 7.8 mM (0.075%, v/v). As a marker of lipid peroxidation, the amount of malondialdehyde (MDA) present in 0.5 ml of these samples was determined by the 1% phosphoric acid method using the TCA (trichloroacetic acid) soluble fractions (11). The results are expressed as nmoles MDA production/10⁶ cells. As a marker of cell membrane damage, LDH activity in the supernatant of centrifuged samples was measured, and results were expressed as % release of LDH (=100×(LDH activity in supernatant–0 time background)/(total activity–0 time background)).

CCI₄-induced acute hepatic injury model
in rats: TRQ or vitamin E was suspended in 0.2% Tween 80 solution and given p.o. to rats weighing about 200 g daily for 14 days. The same amount of 0.2% Tween 80 solution was given to the other rats. CCl₄ diluted with olive oil (37.5%, v/v) was given to the rats (0.75 ml/kg, i.p.) 4 hr after the last administration of these compounds. The animals were killed 20 hr after the injection of CCl₄. Serum was obtained from arterial blood. The liver was washed with 1.15% KCl solution through the portal vein, excised and homogenized in ten volumes of the same solution. Lipid peroxidation in the liver homogenates was quantified by the 1% phosphoric acid method (11). All manipulations were done rapidly on ice to avoid further peroxidation. Glutamic oxaloacetic transaminase (GOT, EC 2.6.1.1) and glutamic pyruvic transaminase (GPT, EC 2.6.1.2) in the serum were measured using commercial test kits (GOT-UV and GPT-UV, Wako Pure Chemicals, Osaka, Japan).

Results

CCI₄-induced enzyme leakage from isolated rat hepatocytes—effect of TRQ pretreatment: It is known that addition of CCl₄ to isolated hepatocytes results in cell membrane fragility, enzyme leakage and eventual cell death (12). Figure 2 shows the dependence of enzyme leakage from the incubated hepatocytes on CCl₄ concentration in the dishes and also on incubation time. The effect of TRQ on the CCl₄-induced enzyme leakage was investigated by pretreatment of rats with TRQ (200 mg/kg/day, p.o., 7 days). The ratio of LDH release from the cells of TRQ-pretreated rats was significantly less than that in control rats (Fig. 2).

CCI₄-stimulated lipid peroxidation in isolated rat hepatocytes—effect of TRQ addition: In order to investigate the action of TRQ on hepatocyte membrane, lipid peroxidation in isolated hepatocytes was studied. The amount of TBA-reactive substances in the TCA soluble fraction of hepatocytes was measured as a marker of lipid peroxidation. The TBA-reactive substances are thought to be MDA and its precursors which separately exist in the cells (11, 13).

The rate of MDA production was accelerated after the addition of 7.8 mM CCl₄ (Fig. 3A). TRQ reduced its rate in a dose-dependent manner, and it completely prevented CCl₄-stimulated lipid peroxidation at a concentration of 33 μM.

Release of LDH from the cells was notably increased by CCl₄ addition (Fig. 3B). TRQ at 33 μM significantly suppressed CCl₄-induced LDH release.

Table 1 shows a comparison between TRQ and vitamin E about the inhibitory effect after CCl₄ addition. With respect to lipid peroxidation, suppressive effects on MDA production of the two compounds were almost equal at 10 and 33 μM. On the other hand, the suppressive effect of TRQ on LDH release was more than twice as strong as vitamin E.

CCI₄-induced acute hepatic injury model in rats: A single administration of CCl₄ (0.75 ml/kg, p.o.) caused a five-fold increase of in vivo lipid peroxidation in the liver (Table 2). In contrast, a reduction of 37% in the lipid peroxidation was obtained by TRQ (100 mg/kg) pretreatment for 14 days prior to CCl₄ treatment. A 63% reduction was observed in
vitamin E (25 mg/kg) pretreated rats. In these experiments, it was proved that the TBA reaction itself was not influenced directly by these two compounds (data not shown). CCl\(_4\) also caused severe hepatocellular damage, with nine- and seventeen-fold increase in serum GOT and GPT, respectively (Table 2). TRQ showed about 40\% reduction of the transaminases increase. Though the reduction of GPT was 37\% in vitamin E receiving animals, only 23\% reduction was observed in the GOT.

**Discussion**

The hepatocytes isolated from TRQ-treated rats showed less leakage of LDH than those from vehicle-treated rats after the addition of CCl\(_4\) as shown in Fig. 2. These ex vivo data clearly prove that TRQ administration suppressed the membrane breakdown in the hepatocytes. It is likely that TRQ displayed its activity by existing in the hepatocytes; actually, TRQ was proved to have high affinity for the liver when it was given p.o. to rats (data not shown).

The mechanism of the hepatotoxicity of CCl\(_4\) is thought of as a continuous reaction. The initial stage is the production of a trichloromethyl radical (•CCl\(_3\)) by a specific form of cytochrome P-450 in liver microsomes (14, 15). It is known that this radical is extremely reactive and then peroxidizes microsomal lipids (6), and/or covalently binds to microsomal lipids and proteins (16, 17).

### Table 1. Inhibition of CCl\(_4\)-induced MDA production and LDH release by TRQ and vitamin E*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (µM)</th>
<th>MDA production (nmol/10^6 cells)</th>
<th>LDH release (%)</th>
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<tr>
<td>None</td>
<td>—</td>
<td>0.01±0.06</td>
<td>2.8±1.3</td>
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<tr>
<td>TRQ</td>
<td>0</td>
<td>4.56±0.05</td>
<td>31.8±2.1</td>
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<td></td>
<td>3.3</td>
<td>2.82±0.17 (38)</td>
<td>20.0±2.5 (41)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.34±0.11 (49)</td>
<td>18.0±2.1 (46)</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>1.52±0.02 (67)</td>
<td>11.8±4.2 (69)</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0</td>
<td>3.72±0.62</td>
<td>33.8±2.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.01±0.46 (46)</td>
<td>27.5±1.7 (20)</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>1.50±0.17 (60)</td>
<td>25.0±1.4 (28)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.06±0.07 (72)</td>
<td>23.5±2.2 (33)</td>
</tr>
</tbody>
</table>

*Hepatocytes (1×10^6 cells/ml) prepared from phenobarbital-treated rats were incubated for one hour with test compounds in the concentrations tabulated above. The cells were then exposed to CCl\(_4\) (7.8 mM). After 2 hr of further incubation, the increase in MDA production and % release of LDH was determined as indicated in Materials and Methods. Values in parentheses indicate percentage figures for reduction of MDA production or LDH release. Each value is the mean±S.E.M. of three flasks.
The early stages of CCl₄ metabolism are followed by pathological changes such as triglyceride accumulation, polyribosomal disaggregation, depression of protein synthesis, cell membrane breakdown, and eventual cell death (15).

Table 2. Effect of TRO and vitamin E on the liver lipid peroxides and the serum transaminases*

<table>
<thead>
<tr>
<th>Group</th>
<th>Compound</th>
<th>Lipid peroxides (nmol/g liver)</th>
<th>GOT (IU/l)</th>
<th>GPT (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreatment</td>
<td>0 mg/kg</td>
<td>-</td>
<td>45.6±3.6***</td>
<td>9.3±0.6***</td>
</tr>
<tr>
<td>CCl₄ control</td>
<td>0 mg/kg</td>
<td>+</td>
<td>389.9±29.8</td>
<td>161.7±15.2</td>
</tr>
<tr>
<td>TRQ</td>
<td>25 mg/kg</td>
<td>+</td>
<td>292.6±25.4* (23)</td>
<td>276.0±39.1* (33)</td>
</tr>
<tr>
<td></td>
<td>50 mg/kg</td>
<td>+</td>
<td>293.6±28.5* (22)</td>
<td>286.0±30.7* (30)</td>
</tr>
<tr>
<td></td>
<td>100 mg/kg</td>
<td>+</td>
<td>253.6±33.8* (37)</td>
<td>237.6±25.1** (44)</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>25 mg/kg</td>
<td>+</td>
<td>181.7±26.1*** (63)</td>
<td>311.3±20.4* (23)</td>
</tr>
</tbody>
</table>

*Each value is the mean±S.E.M. of 10 rats (only the non-treated group is 8 rats). Values in parentheses indicate percent figures for reduction of lipid peroxides or serum transaminases. Key: (*)P<0.1, (**)P<0.05 (***P<0.01 and (****)P<0.001, compared with the CCl₄-treated control group (Student's t-test).

SKF 525-A which was known to prevent the hepatotoxicity of CCl₄ was shown to reduce the amount of cytochrome P-450 (17). However, it was revealed that the amount of cytochrome P-450 did not change in TRQ receiving animals (18). Thus, the membrane protecting action of TRQ was not dependent on the inhibition of cytochrome P-450.

While there are many arguments about which contributes most importantly to the early stages of CCl₄ hepatotoxicity, lipid peroxidation or covalent binding of -CCl₃ (19), several reports show that suppression of lipid peroxidation results in protection of isolated hepatocytes against cell membrane breakdown in vitro (20, 21). In order to know if an action point of TRQ is ascribed to the suppression of lipid peroxidation, we investigated the effect of TRQ on cell membrane peroxidation in comparison with vitamin E, an endogeneous antioxidant. When CCl₄ was added to freshly isolated hepatocytes to stimulate the lipid peroxidation, the MDA production was strongly prevented in the cells preincubated with TRQ (Fig. 3A).

As shown in Fig. 3B, the addition of TRQ to the medium suppressed the cell membrane breakdown, suggesting that there is a correlation between membrane peroxidation and membrane breakdown. However, it is difficult to explain the CCl₄-induced cell membrane breakdown only by lipid peroxidation in the membrane, because complete prevention against lipid peroxidation at 33 µM of TRQ did not lead to the complete suppression of enzyme leakage.

The inhibitory action of TRQ on lipid peroxidation was almost the same as that of vitamin E (Table 1). On the other hand, the suppression of TRQ on enzyme leakage was more than twice as strong as vitamin E. These data mean that TRQ has not only an antioxidative activity on hepatocyte membrane but also another action which contributes to membrane protection.

It is well-known that CCl₄-induced lipid peroxidation also occurs in vivo. (6). CCl₄ administration to the rats remarkably increased lipid peroxides in the liver measured according to the 1% phosphoric acid method (Table 2). This method represents the combined status of tissue peroxidation including several antioxidative and prooxidative factors (11, 13). TRQ (100 mg/kg) significantly suppressed the increase in total lipid peroxides as well as enzyme leakage. So it is thought that the membrane protecting action of TRQ depends on its suppressive effect against lipid peroxidation in vivo. However, the protection against increase of GOT by vitamin E was weaker than that by TRQ, in spite of its stronger inhibition of lipid peroxidation. From these data, similar to the results of isolated hepatocytes, it is suggested that lipid peroxidation is one of the major causes of cell injury but never the
only cause (19); in addition, the data indicate that TRQ has a different action from vitamin E. We previously reported that TRQ also suppressed the increase of serum transaminases in acute hepatic injured rats induced by D-galactosamine and allyl alcohol (5), which were not accompanied by membrane lipid peroxidation (data not shown). Smith et al. reported that vitamin E had no protective effect on a cytotoxicity induced by bromobenzene, a hepatotoxin which produced no lipid peroxide in freshly isolated hepatocytes (21). From these results, it is suggested that the membrane protecting action of TRQ is partially derived from its suppressive effect on lipid peroxidation, but "another action" may also play an important role in protecting the fragile membrane. Further investigation is necessary to obtain more information about this "other action".

References