Effect of Cycloheximide on Increased Aspartate Aminotransferase in Carbon Tetrachloride Hepatotoxicity*

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ABSTRACT

The previously reported increases in liver and serum aspartate aminotransferase (ASAT) activities and liver protein content 24 hours after the administration of carbon tetrachloride (CCl₄) were reduced by administering multiple doses of the protein synthesis inhibitor cycloheximide (CH). Liver ASAT and protein content were reduced to saline-injected control levels, and the serum ASAT increase was reduced by 45.0 percent in rats given CH. Although there are morphological features of severe hepatotoxicity in the cycloheximide-carbon tetrachloride-injected rats, cycloheximide does reduce the severity of these lesions and the regenerative response. These findings lend some support to the hypotheses that (1) the increase in liver ASAT activity and protein content after CCl₄ is due to increased synthesis and (2) the increase in serum ASAT after CCl₄ is most likely due to a combination of increased synthesis and leakage from necrotic and damaged cells.

Introduction

The release of intracellular enzymes into the circulatory system from damaged cells following myocardial infarction was first discovered in 1954. Specific reference to serum aspartate aminotransferase (ASAT), EC 2.6.1.1., release in response to hepatocellular insult was reported the following year. Several early reports on the effect of carbon tetrachloride (CCl₄) toxicity indicated that there was a reduction in liver enzyme activity. A more recent report has suggested that the liver enzyme levels actually increase when the reference standard is changed to accommodate the hepatomegaly and edema seen following CCl₄ poisoning.

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The increase in serum enzyme seen following hepatocellular injury has classically been explained as being due to tissue necrosis and subsequent leakage of enzyme from necrotic cells into the plasma.\textsuperscript{3,6,19,20} The recent report from this laboratory has suggested that at least a portion of this increase is a result of increased enzyme synthesis in response to hepatocellular injury.

The purpose of the present study was to determine to what extent increased enzyme synthesis contributes to the rise in liver and serum ASAT activities following acute CCl\textsubscript{4} poisoning.

**Materials and Methods**

Male albino rats (Sprague-Dawley) 140 to 210 g were injected subcutaneously with 1.0 mg per kg cycloheximide (CH)* in distilled water or with an equivalent volume of isotonic saline. These animals then received an i.p. injection of 0.75 ml per kg CCl\textsubscript{4}† or an equivalent volume of saline 40 min following the initial injection. Subsequent s.c. injections of 1.0 mg per kg CH or equivalent saline volumes for controls were administered at four and 12 hrs following CCl\textsubscript{4} administration. A final dose of 0.5 mg per kg cycloheximide was given 20 hrs post-CCl\textsubscript{4}. Food was removed at the time of initial injection, but water was permitted ad libitum.

Prior to sacrifice, the rats were evaluated and assigned a grade as follows: (0) normal rat; (1+) rat has rough coat and eyelids appear heavy; (2+) rat can walk, but movements are sluggish; (3+) rat unable to walk or stand, can crawl away to avoid being disturbed; (4+) rat is unable to move, not responsive to external stimuli.

The rats were then sacrificed 24 hrs following CCl\textsubscript{4} injection by decapitation. Blood samples were collected, the liver excised and weighed, and a one g sample removed and minced. This minced liver tissue was added to one ml cold sucrose-ethylene diamine tetraacetic acid (EDTA) solution (250 mM sucrose, 1 mM EDTA, pH 7.8), and homogenized with three one minute cycles using a Polytron tissue homogenizer.‡ This homogenate was diluted to 10 ml with cold sucrose-EDTA solution, dispensed into 1.5 ml aliquots, and stored at +4° until time of assay, which did not exceed 10 hrs after sacrifice.

Enzyme activity was determined by the method of Karmen\textsuperscript{8} as modified\textsuperscript{1,5} and activated by the addition of coenzyme pyridoxal phosphate.\textsuperscript{15} The assays were performed using Dow substrates and reconstituting reagents on the CentrifiChem\textsuperscript{®} clinical analyzer.§ Liver enzyme activity is expressed as International Units (IU) per total liver per gram initial body weight (i.e., at the time of first injection) while serum enzyme activity is expressed as IU per liter of serum. The total protein content of the liver homogenate samples was determined by the Lowry method.\textsuperscript{11} Protein content is expressed as mg of protein per total liver per gram initial body weight.

Samples of liver tissue from the left lobe were formalin-fixed and retained for morphological examination. This liver tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The slides were then coded and examined by three different pathologists who assigned a score from 0 to 4+, 0 being normal and 4+ being most severe. These individual values were then averaged to obtain a consensus value. These consensus values for each pair of rats were then compared using Student’s t-test for paired observations.\textsuperscript{17} The scores were assigned in the following categories (1) extent of involvement; (2) necrosis; (3)

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* Sigma.
† Matheson, Coleman, and Bell, Spectroquality.
‡ Brinkmann Instruments.
§ Union Carbide Corporation.
number of balloon cells; (4) fatty change; (5) inflammation; (6) amount of regeneration. In addition, an average of all of these individual values, except for regeneration, was obtained. This average value is referred to as the severity index. The following criteria were considered as evidence of regeneration: increased size of cells, nuclei, and nucleoli; increased number of nuclei per cell (polyplody); increased number of nucleoli per nucleus; increased number of mitoses and chromatin activity.

The rats were tested in groups of three to minimize the effects of variations in body weight and experimental conditions. The injections were always given at the same time of day to avoid diurnal variations. The t-test for paired observations was again used to test for significance between groups for enzyme and protein values. Fisher's exact test was used for testing difference in mortality between groups.²

Results

The results of the various treatments on ASAT levels in rat liver tissue are presented in figure 1. Liver ASAT activity was significantly increased (p < 0.001) by 27.9 percent in the CCl₄-injected rats vs. the saline-injected controls. Rats that received CCl₄ and cycloheximide had significantly lower (p < 0.001) liver enzyme levels than rats receiving CCl₄ alone. These lower values did not differ significantly from the liver enzyme values found in the control group.

A highly significant (p < 0.001) increase in serum ASAT activity is observed in rats that received CCl₄ alone when compared to controls (figure 2). When cycloheximide was given in conjunction with CCl₄, a significant decrease (p < 0.001) in serum ASAT response to CCl₄ was observed. A comparison of serum enzyme values from saline-treated controls with rats receiving both drugs revealed that while there was still a significant increase (p < 0.001) in ASAT activity in the treated group, there was a 45 percent reduction from the CCl₄ activity levels.

The livers of the CCl₄-treated rats had significantly elevated (p < 0.01) protein content as compared to saline-injected controls (figure 3). The liver protein content of rats receiving both drugs was significantly lower (p < 0.01) than rats receiving only CCl₄, but it did not differ significantly from the saline-injected controls.
Rats injected with CCl₄ had a significant decrease (p < 0.05) in enzyme specific activity (778 IU per mg protein) compared to the saline group (875 IU per mg protein). There was no significant difference between the saline-treated rats and those receiving both drugs.

Morphological studies demonstrate that the saline-injected control rats displayed no pathological changes while both the CCl₄ and CCl₄ + cycloheximide groups displayed numerous and significant liver lesions. In comparing the results of the three independent assessments (table I), it was revealed that the degree of necrosis, number of balloon cells, degree of fatty change, overall severity of damage, and the amount of regeneration were significantly reduced in the rats that received both drugs compared to those that received CCl₄ only. The effects of CCl₄ and CH on mortality and toxicity scores are shown in table II.

**Discussion**

These data lend some support to the previously presented hypothesis¹³ that increased synthesis is responsible for the increased liver ASAT activity and for a significant proportion of the increased serum activity 24 hrs after injection of CCl₄. The increased liver ASAT and protein content after CCl₄ were reduced to control levels by multiple injections of the protein synthesis inhibitor cycloheximide (figures 1 and 3). The increased serum ASAT activity after CCl₄ was reduced by 45.0 percent with CH. Cycloheximide also reduced the severity of the CCl₄-induced morphological changes but not enough to explain the reduction of liver enzyme activity and protein content to control levels and the reduction of serum enzyme levels by 45.0 percent. The Severity Index (table I) was reduced by 28.4 percent, and necrosis alone was reduced by 35.7 percent. Linear regression analysis revealed that the serum enzyme and morphological decreases were not correlated. Thus, the decrease in serum activity was too great to be ex-

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**TABLE I**

Morphological Comparison of Rat Livers*

<table>
<thead>
<tr>
<th>Group</th>
<th>Extent (Amount of Lobule)</th>
<th>Necrosis</th>
<th>Balloon Cells</th>
<th>Fatty Change</th>
<th>Inflammation</th>
<th>Severity Index</th>
<th>Regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCl₄</td>
<td>1.71</td>
<td>1.40</td>
<td>2.36</td>
<td>1.81</td>
<td>1.08</td>
<td>1.69</td>
<td>3.03</td>
</tr>
<tr>
<td>CCl₄ + CH</td>
<td>1.46</td>
<td>0.90</td>
<td>1.87</td>
<td>0.85</td>
<td>0.97</td>
<td>1.21</td>
<td>1.44</td>
</tr>
<tr>
<td>Percent change</td>
<td>-14.6</td>
<td>-35.7</td>
<td>-20.7</td>
<td>-53.1</td>
<td>-10.2</td>
<td>-28.4</td>
<td>-52.5</td>
</tr>
<tr>
<td>p</td>
<td>N.S.</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>N.S.</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Values represent the means of the consensus values for 12 rats in each group. Consensus values obtained by averaging grades. The range of grades for each of the above categories is 0 - 4⁺, 0 = no pathological changes and 4⁺ = most severe changes. Severity Index is an average of Extent, Necrosis, Balloon Cells, Fatty Change, and Inflammation categories.
TABLE II
Mortality Rate and Average Toxicity for Rats*

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. Rats</th>
<th>No. Injected</th>
<th>No. Dead</th>
<th>Percent Mortality</th>
<th>Average Toxicity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Saline</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>CCl4</td>
<td>37</td>
<td>3</td>
<td>8.1</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>CCl4 + CH</td>
<td>53</td>
<td>10</td>
<td>18.9</td>
<td>2.85</td>
<td></td>
</tr>
</tbody>
</table>

P < 0.04 < 0.001

Treatment B vs. C

Toxicity scores range from (0) for normal rat to (4+) for most toxic.

plained by the protective morphological effect of cycloheximide alone. In contrast to the protective effect of cycloheximide on the CCl₄-induced morphological liver lesions, cycloheximide increased the mortality rate and toxicity scores when used with CCl₄ compared to rats given only CCl₄ (table II).

Several earlier reports suggested that rat liver ASAT activity decreased following CCl₄. Some investigators attempted to explain these apparent decreases by postulating that enzyme "leaked" out of the necrotic hepatocytes resulting in decreased liver activity and increased serum enzyme activity. The decrease in activity per gram wet weight after CCl₄ reported by others, however, is due primarily to hepatomegaly as a consequence of edema and fatty change with dilution of enzyme rather than to "leakage" of enzyme from necrotic cells as is commonly held. When total liver per gram initial body weight is used as the reference standard, ASAT activity is increased 24 hrs after CCl₄.

In the previous report, it was found that specific activity (IU per mg protein) was unchanged 24 hrs after CCl₄ since it was found that liver protein content, expressed as mg protein per total liver per gram initial body weight, was increased to approximately the same degree as enzyme activity. In the present study using 0.75 ml CCl₄ per kg body weight, rather than 1.00 ml, plus four s.c. saline injections, the specific activity decreased significantly by 11.1 percent. This contrasts with the actual increase in ASAT activity after CCl₄ of 27.9 percent when expressed as IU per total liver per g initial body weight in the CCl₄-injected group when compared to the saline controls. This decrease in specific activity was due to the larger increase in total protein content, 49.6 percent, compared to the ASAT increase of 27.9 percent and not to decreased enzyme activity. This points out the erroneous conclusions that can be drawn if one uses protein as the reference standard for enzyme activity.

Popp, Shinozuka, and Farber reported that cycloheximide (1.2 mg per kg given three times at six hr intervals beginning 10 min before CCl₄ and which was given at doses of either 1.25 or 0.63 ml of CCl₄ per kg body weight) completely prevented centrilobular necrosis at least for the first 24 hrs. In the present study, although the liver damage was less severe in the group that received cycloheximide + CCl₄, the severity of the lesions in the cycloheximide + CCl₄ group, including necrosis, was still considerable (table I) at 24 hrs. Thus, while cycloheximide provides some protective effect to the morphological effects of CCl₄-induced liver injury in this study, this effect is not enough to explain the striking decrease in liver enzyme activity and protein content to control levels. Most of this decrease is likely due to inhibition of protein and enzyme synthesis. This decrease in synthesis is also the most likely cause of much of the 45 percent decrease in serum enzyme activity which also correlates well with the decrease in regenerative activity (table I).

Lindstrom and Anders observed a 43.8 percent reduction of the CCl₄-induced increase in serum ASAT using cycloheximide, similar to the 45.0 percent reduction observed in this report. They gave a single injection of 1.5 mg per kg
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CH s.c. 0.5 hr prior to the injection of 0.1 ml per kg CCl₄ (10 percent corn oil, i.p.) and sacrificed 24 hrs after CCl₄. They interpreted the decrease in serum ASAT as being due to the effect of cycloheximide in reducing the severity of CCl₄-induced hepatotoxicity alone. They did not measure liver ASAT activity. Our interpretation is that an important additional explanation of the decrease in serum ASAT is inhibition of enzyme synthesis by cycloheximide. Our explanation is confirmed by the decreased liver enzyme activity and protein contents to control levels using cycloheximide + CCl₄.

The mortality rate was significantly increased (p < 0.04) in the cycloheximide + CCl₄ group (18.8 percent) as compared to the CCl₄ group (8.1 percent) (table II). This would suggest that cycloheximide, while lessening the morphological evidence of liver damage, increases the overall toxicity of CCl₄. This is confirmed by observations of increased signs and symptoms of toxicity in the cycloheximide + CCl₄ group, i.e., increased lethargy, decreased response to stimuli, ataxia, tachypnea, ptosis of the eyelids, rough fur, etc. (table II).

It has been shown¹⁶ that a dose of 1.0 mg per kg of cycloheximide will depress rat liver protein synthesis at two hrs after administration. If no additional doses of drug are given, protein synthesis will return to normal levels by 24 hrs postinjection. Other investigators¹⁸ have shown that a dose of 1.5 mg per kg will depress protein synthesis by more than 50 percent at seven hrs after administration. The multiple doses of cycloheximide used in this study were based on our preliminary experiments and designed to maintain the depression of protein and enzyme activity over a 24 hr period. The reduced liver protein content of rats that received both drugs compared to those that received CCl₄ alone indicates that liver protein content (figure 3) and liver enzyme activity (figure 1) were reduced as anticipated.

The hypothesis was previously presented¹³ that increased synthesis of ASAT by viable, including regenerating, hepatocytes in response to injury by CCl₄ is responsible for the increase in liver ASAT and possibly for a significant proportion of the increased serum ASAT activity as well. The data presented here lend some support to this hypothesis which remains, however, unproven. A significant part of the decrease in serum activity in the cycloheximide + CCl₄ group compared to the CCl₄-alone group is also due to the decreased severity of the lesion and decreased regenerative response when cycloheximide is used. “Leakage” of enzyme from necrotic hepatocytes and from viable hepatocytes through damaged cell membranes is considered likely to be the source of most of the remaining (55.0 percent) increase in serum activity after CCl₄.

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References

5. Henry, R. J., Chiamori, N., Golub, O. J., and Berkman, S.: Revised spectrophotometric methods for the determination of glutamic-oxal-


