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EFFECTS OF ADENOSINE ON LIVER CELL DAMAGE INDUCED BY CARBON TETRACHLORIDE

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Abstract—Adenosine administration delayed the fatty liver and cell necrosis induced by carbon tetrachloride without affecting the action of the hepatotoxin on protein synthesis and liver triacylglycerol release. Adenosine produced a drastic antilipolytic effect accompanied by a decrease in the incorporation of [1-^{14}C]palmitic acid into triacylglycerols and free fatty acids of the liver. Furthermore, a decrease in the serum levels of ketone bodies was observed at early times. The nucleoside also avoided the release of intracellular enzymes and prevented the lipid peroxidation produced by carbon tetrachloride during the 4 hr of treatment. The protective action of adenosine was transient, lasting 3–4 hr, probably the time required to be metabolized. The results suggest that the antilipolytic effect of the nucleoside, the inhibition of hepatic fatty acid metabolism, and the decrease in carbon tetrachloride-induced lipoperoxidation that it produced are involved in the delayed acute hepatotoxicity induced by carbon tetrachloride.

Carbon tetrachloride (CCl_4) induces fatty liver and liver cell necrosis [1–3]. The precise sequence of events that follows CCl_4 administration and leads to these pathological states is not well known. However, several effects of the hepatotoxin seem to play a significant role in inducing liver cell damage such as inhibition of triacylglycerol (TGS) release from the liver [4] and increased lipoperoxidation in membranes [5] whose structural integrity is necessary for lipoprotein release [6], finally resulting in liver TGS accumulation and destruction of the liver cell.

Previous work in our laboratory has shown that the administration of adenosine almost completely prevents the induction of fatty liver by ethanol [7, 8] and cycloheximide [9]. The main purpose of the present study was to determine whether the administration of the nucleoside also protects against the fatty liver and the necrotizing action produced by an acute administration of CCl_4.

MATERIALS AND METHODS

Adenosine, 2-thiobarbituric acid, and zeolite were obtained from the Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Palmitic acid (sodium salt, 17.8 mCi/mmole) and [1-^{14}C]leucine (59 mCi/mmole) were purchased from the International Chemical & Nuclear Corp. (Irvine, CA) and the Radiochemical Center (Amersham, U.K.) respectively. Coenzymes were obtained from Boehringer und Sohene (Mannheim). Silica gel 60 F254 and carbon tetrachloride (analytical grade) were obtained from the Merck Co. Other chemical reagents used were of the best quality available.

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The experiments were performed with male Wistar rats weighing between 140 and 200 g and fasted for 16–20 hr. Carbon tetrachloride was diluted with vegetal oil (1:4, v/v) and administered through a stomach tube at a dose of 2.5 ml/kg body weight. Thereafter, animals were injected intraperitoneally with saline (0.85% NaCl) or saline-suspended adenosine (200 mg/kg body weight) at pH 7.4.

Four groups of animals were used for each experiment, rats receiving (a) oil + saline, (b) oil + adenosine, (c) carbon tetrachloride + saline, and (d) carbon tetrachloride + adenosine.

Hepatic triacylglycerols and free fatty acids were determined by the methods of Butler *et al.* [10] and Novak [11] respectively. Serum triacylglycerols and free fatty acids were measured by the methods of Van Handel and Zilversmit [12] and Dole and Meinertz [13] respectively. Blood ketone bodies were quantified enzymatically: 3-β-hydroxybutyrate according to Williamson and Mellamby [14] and acetoacetate according to Mellamby and Williamson [15].

To study hepatic protein synthesis, animals were injected with radioactive leucine (50 μCi/kg body wt) 30 min before being killed. The incorporation of the labeled aminoacid into liver protein and the acid soluble radioactivity were determined as described Jazcilevich and Villa-Treviño [16].

Incorporation of [1-^{14}C]Palmitate into total liver lipids was determined as follows: a pulse of labeled fatty acid (10 μCi/kg body wt) was given 15 min before sacrifice, and a hepatic sample was removed, weighed and homogenized. Lipids were extracted by the method of Folch *et al.* [17], and radioactivity in the lipid extracts was counted as described previously [18].

Thin-layer chromatography [19] was used for the separation of hepatic lipids. Triacylglycerols and free fatty acids were visualized with iodine vapor staining. The radioactivity of the lipid fractions was counted

as previously described [18]. For the separation of phospholipids the method described by García-Sáinz and Fain [20] was employed with quantification of inorganic phosphate by the method of Ames and Dubin [21].

The activities of serum alanine-aminotransferase (SALT, EC 2.6.1.2) and aspartate-aminotransferase (SAST, EC 2.6.1.1) were measured according to the methods of Reitman and Frankel [22]. Serum glutamic dehydrogenase (SGDH, EC 1.4.1.3) activity was measured by the method of Schmidt [23]: one unit reduced 1.09 μmoles of α-ketoglutarate/ml/hr [24].

Lipoperoxidation was quantified by the thiobarbituric acid method [25] using liver homogenates in bidistilled water (1:9, w/v) as enzyme source; all manipulations were made on ice and rapidly to avoid peroxidation. Some modifications to the original method were introduced: a sample of the homogenate (approximately 1 mg protein) was incubated for 30 min at 37° in 1 ml of 0.15 M Tris, pH 7.4; incubation was ended by adding 1.5 ml of 20% acetic acid (adjusted to pH 2.5 with KOH) and 1.5 ml of 0.8% thiobarbituric acid. The samples were kept for 45 min in a boiling water bath and 1 ml of 2% KCl was added at the end of the incubation to each sample. The colored complex formed was extracted with butanol–pyridin (15:1, v/v) and detected at 532 nm. The extinction coefficient of malonaldehyde color complex was $1.56 \times 10^5\ cm^{-1}\ M^{-1}$ [26, 27].

Protein was determined by the biuret method [28]. Statistical significance between comparable groups was determined by Student's *t*-test.

RESULTS

Administration of CCl_4 markedly increased the amount of hepatic TGS (Fig. 1); simultaneous administration of adenosine diminished this effect. The difference between the group treated with CCl_4 + saline and the animals treated with CCl_4 + adenosine was statistically significant at 4, 6 and 8 hr ($P < 0.02$, $P < 0.01$ and $P < 0.05$ respectively). Adenosine alone did not change the hepatic content of TGS (Fig. 1).

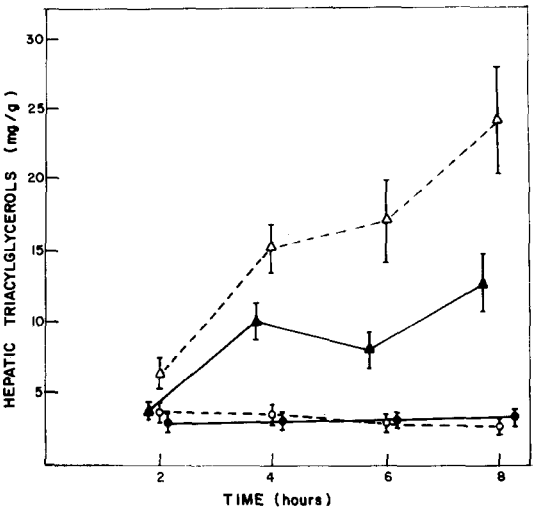

Fig. 1. Time-course of the effect of carbon tetrachloride and adenosine on the hepatic concentration of triacylglycerols. Key (○----○) oil + saline, (●——●) oil + adenosine, (△----△) CCl_4 + saline and (▲——▲) CCl_4 + adenosine. Vertical bars represent the mean ± standard error of at least five independent observations.

The possibility that the antilipolytic action of adenosine [29] could be involved in the prevention of CCl_4-induced fatty liver was tested. The results are shown in Table 1. After 1 hr of treatment, the serum free fatty acids (FFA) level was strongly decreased in the group of animals treated with adenosine alone and this effect was even bigger in animals treated with CCl_4. This antilipolytic effect of the nucleoside was less evident at later times. Eight

Table 1. Effects of carbon tetrachloride and adenosine on serum lipids*

Treatment	Time (hr)	Free fatty acids (% of control)	Triacylglycerols (% of control)
Oil + saline	1	100	100
	4		
	8		
Oil + adenosine	1	31 ± 4†	89 ± 6
	4	86 ± 16	89 ± 5
	8	93 ± 7	97 ± 8
CCl_4 + saline	1	107 ± 16	102 ± 10
	4	91 ± 12	75 ± 6†
	8	140 ± 13‡	54 ± 10†
CCl_4 + adenosine	1	15 ± 2†§	80 ± 1†
	4	86 ± 11	86 ± 4†
	8	124 ± 11	39 ± 5†

* Control values were 444.1 ± 77.3 μmoles/l and 79.5 ± 2.4 mg/100 ml for FFA and TGS respectively. No difference in control values was observed at all times measured. Results shown are the mean ± S.E.M. of at least five independent observations.

† $P < 0.02$, compared to oil + saline group.

‡ $P < 0.05$, compared to oil + saline group.

§ $P < 0.005$, compared to CCl_4 + saline group.

Table 2. Effect of carbon tetrachloride and adenosine on the hepatic incorporation of [1-^{14}C]palmitate into total lipids*

Treatment	[^{14}C]Palmitate	
	Incorporation (cpm/g liver wet wt)	Specific activity (cpm/mg lipid)
Oil + saline	5109 ± 382	233 ± 34
Oil + adenosine	5370 ± 862	191 ± 46
CCl_4 + saline	8687 ± 793†	267 ± 19
CCl_4 + adenosine	5114 ± 1059‡	171 ± 34‡

* Determinations were made 1 hr after treatment. The results are expressed as the mean ± S.E.M. of five independent experiments.
† $P < 0.005$, compared to oil + saline group.
‡ $P < 0.05$, compared to CCl_4 + saline group.

hours after treatment, CCl_4 increased significantly the serum FFA level and adenosine was unable to block this effect (Table 1).

Another possible mechanism for preventing CCl_4-induced fatty liver is an increase in the release of TGS by the organ; therefore, TGS serum level was quantified (Table 1). As previously shown [4, 30, 31] and as reflected by the serum levels, CCl_4 impaired TGS secretion (Table 1). However, adenosine was unable to prevent this effect of CCl_4 (Table 1). Adenosine is known to inhibit the initial step in FFA metabolism in the liver, i.e. the formation of the CoA thioester [32]. Therefore, we tested the possibility that adenosine might act by affecting both FFA incorporation and its metabolism.

CCl_4 significantly increased [1-^{14}C]palmitic acid incorporation into the liver lipids (Table 2); however, no change in the specific activity was found in this group as compared to the control group, indicating that the increase in radioactivity merely reflects lipid accumulation specially on TGS and phospholipids (Table 3).

The decrease in specific activity produced by adenosine administration indicates that less palmitic acid was incorporated, as it was clearly observed at 1 hr of treatment (Table 3). At 4 and 8 hr, the effect of the hepatotoxin on the palmitic acid incorporation into the liver lipids was also observed (10 and 26% of increment compared to control group respectively). At both times, adenosine was able totally to prevent this effect.

Consistently with adenosine inhibition of the liver FFA uptake, we observed that the nucleoside strongly reduced FFA oxidation as evidenced by the dramatic decrease in the serum ketone body level (Fig. 2).

It is well known that CCl_4 administration produces an inhibition of hepatic protein synthesis [33, 34]. Adenosine did not affect this toxic action of CCl_4 (data not shown). However, it did provide protection against CCl_4 liver cell necrosis during the first 4 hr after the treatment. This effect was evidenced when SALT and SAST serum levels were determined (Fig. 3). At later times, the serum activities of both enzymes increased even in those animals treated with the nucleoside; parallel increases in enzyme activities were observed, but the difference between the CCl_4 + saline group and the CCl_4 + adenosine group was statistically significant at all times tested (Fig. 3).

SGDH serum activity has been reported as a marker of liver cell necrosis [35, 36]; this activity was therefore determined and the results showed the same profile as both SALT and SAST activities. The values obtained at 4 hr for SGDH were 142 ± 28 and 195 ± 43 mUnits/ml for oil + saline and oil + adenosine respectively; 12 hr after the treatment no change in the control values was found. Four hours after the treatment, SGDH activity in the CCl_4 + saline and CCl_4 + adenosine groups was 378 ± 42 and 142 ± 34 mUnits/ml respectively, whereas at 12 hr CCl_4 + saline and CCl_4 + adenosine groups showed levels of 2301 ± 707 and 1434 ± 685 mUnits/ml respectively.

This action of the nucleoside lead us to study a possible effect of adenosine on CCl_4-induced lipoperoxidation. The results are shown in Fig. 4. Maximal lipoperoxidation induced by the hepatotoxin was observed 15 min after the treatment, as previously reported [37], and a clear decrease was observed in the presence of adenosine. A small but consistent increase in lipoperoxidative activity was observed in the control groups treated with oil and saline or adenosine as compared to untreated ani-

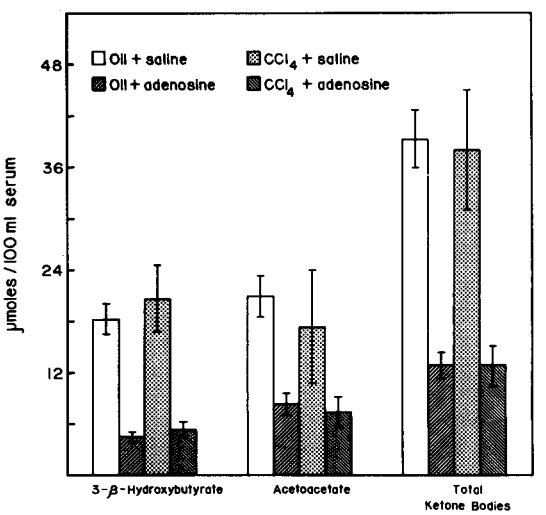

Fig. 2. Serum levels of ketone bodies. Total ketone bodies were calculated as the sum of 3-β-hydroxybutyrate plus acetoacetate. Vertical bars represent the mean ± standard error of at least five animals.

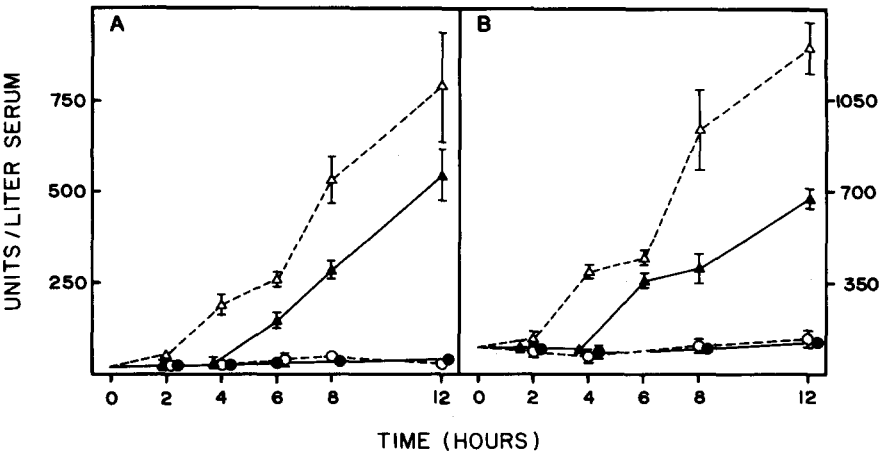

Fig. 3. Time-course of the effect of carbon tetrachloride and adenosine on the serum levels of aminotransferase activities. Panel A shows the SALT activity. Panel B shows the SAST activity. Each point is the average of four to ten independent experiments. Vertical bars represent the standard error. Symbols are defined in the legend of Fig. 1.

mals, probably as a result of the stress induced by the treatment.

DISCUSSION

The present work shows that intraperitoneal administration of adenosine delayed the hepatotoxic effects of acute CCl_4 treatment. Probably the nucleoside effect in the fatty liver was mediated by its early antilipolytic action (Table 1) and by the inhibition of acyl CoA synthesis previously reported [32].

This assumption is supported by the diminution of [1-^{14}C]palmitic acid incorporation into hepatic lipids (FFA and TGS, Tables 2 and 3), and by the decrease in blood ketone bodies (Fig. 2) as compared with the group treated with CCl_4.

At the same time, CCl_4 enhanced liver incorporation of labeled palmitic acid without change in specific activity, suggesting label accumulation. The results are consistent with the work of other authors [38–40]. The effects of adenosine were transient; the nucleoside produced a nearly 2–4 hr delay in CCl_4 action. This effect was also evident as far as protection against liver cell necrosis (Fig. 3). Once the protective action of adenosine disappeared, the hepatotoxicity followed the same pattern observed in the CCl_4 + saline group.

A correlation between the hepatic TGS accumulation and the liver cell necrosis in the CCl_4-treated animals has not been clearly established, mainly because other experimental conditions such as ethionine treatment or choline-deficient diets induce fatty liver without producing necrosis. On the other hand, some drugs such as antihistaminics prevent the necrogenic effects of CCl_4 treatment but do not avoid the increase in liver lipids [41]. In our case, a strong correlation was observed between both events, i.e. the hepatic TGS accumulation and the serum aminotransferase activities taken as an index of necrosis. The correlation coefficients observed were $r = 0.995$ between hepatic TGS and SALT activity and $r = 0.997$ between liver TGS and SAST activity in the CCl_4 + saline group; similar correlation coefficients were observed in the CCl_4 + adenosine group. These results suggest that CCl_4 hepatotoxicity is the same at the beginning of treatment as it is 4 hr after treatment in the nucleoside group. The results also suggest that the nucleoside acts during the primary event of CCl_4 injury.

Several authors have reported that the first step in the CCl_4 liver damage is a lipoperoxidative phenomenon [5, 6]; membrane lipids of the endoplasmic reticulum are rich in polyenoic fatty acids, susceptible to peroxidative decomposition that may cause a disintegration of cell membranes and cell necrosis. The lipoperoxidative action induced by CCl_4 as detected by malonaldehyde (MDA) formation is due to 3-ω-unsaturated fatty acids; this pro-

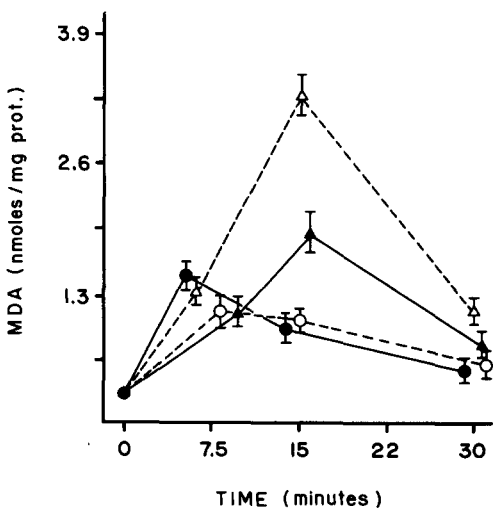

Fig. 4. Effect of carbon tetrachloride and adenosine on endogenous liver lipoperoxidation. Key: (○----○) oil + saline, (●——●) oil + adenosine, (△----△) CCl_4 + saline and (▲——▲) CCl_4 + adenosine. The time zero corresponds to untreated animals. Vertical bars represent the standard error of the average for at least five animals.

Table 3. Distribution of [^{14}C]palmitate among several lipid fractions from livers of carbon tetrachloride- and adenosine-treated rats*

Treatment	[^{14}C]Palmitate					
	Fatty acids		Triacylglycerols		Phospholipids	
	cpm × 10^3/g liver (wet wt)	Specific activity	cpm × 10^3/g liver (wet wt)	Specific activity	cpm × 10^3/g liver (wet wt)	Specific activity
Oil + saline	2.71 ± 0.35	2.24 ± 0.29	1.20 ± 0.24	0.44 ± 0.08	2.22 ± 0.35	68 ± 5
Oil + adenosine	2.69 ± 0.28	1.87 ± 0.28	1.05 ± 0.17	0.37 ± 0.07	2.41 ± 0.45	46 ± 3†
CCl_4 + saline	2.91 ± 0.31	1.73 ± 0.22	1.80 ± 0.35	0.50 ± 0.04	3.18 ± 0.46†	74 ± 10
CCl_4 + adenosine	1.99 ± 0.15‡	0.88 ± 0.10§‖	0.73 ± 0.16‡	0.19 ± 0.04§‖	2.97 ± 0.32	81 ± 9

* Specific activity is expressed as cpm × 10^3/mg lipid. The animals were treated for 1 hr and the results are the mean ± S.E.M. of at least five independent observations.
† $P < 0.05$, compared to oil + saline group.
‡ $P < 0.02$, compared to CCl_4 + saline group.
§ $P < 0.005$, compared to oil + saline group.
‖ $P < 0.001$, compared to CCl_4 + saline group.

cess is of short duration, reaching a peak 15 min after the hepatotoxic treatment, as has been shown previously [37]; in the presence of adenosine a 50% reduction of this phenomenon was observed (Fig. 4). An interesting finding was that 4 hr after treatment, probably at the time that the adenosine effect disappears, endogenous lipoperoxidation was so high in the group treated with CCl_4 + adenosine as to reach the value obtained at 15 min after treatment with CCl_4 + saline; the CCl_4 + adenosine group showed an increased lipoperoxidation value (1.49 ± 0.09 nmoles MDA/mg protein) when compared to that observed in animals treated with oil + saline, oil + adenosine and CCl_4 + saline (0.95 ± 0.09, 0.97 ± 0.03 and 0.87 ± 0.07 nmole MDA/mg protein respectively). This confirms the lag produced by adenosine in the hepatotoxic effects of CCl_4.

Under our conditions, adenosine presented a hypothermic effect, lowering the basal body temperature 2 or 3 degrees centigrade during the first hours of treatment. This finding is important since several authors [42, 43] have shown a blocking in CCl_4 induction of fatty liver and cell necrosis by lowering the body temperature of the rats.

Adenosine action on CCl_4 hepatotoxicity does not seem to be related to the hypothermic action of the nucleoside because its action in fatty liver formation and cell necrosis was observed when the decrease in body temperature was prevented by placing the experimental animals in a room at 37°.

These results are in accordance with previous data in our laboratory. It is clear that adenosine can prevent or delay the induction of fatty liver by several agents such as ethanol, cycloheximide and carbon tetrachloride [7–9]. These hepatotoxic agents have different mechanisms for inducing fatty liver. It is obvious that adenosine does not specifically inhibit each one of these compounds and that its main action occurs at the level of a common pathway which seems to be at the level of acyl-CoA synthetase. However, adenosine does not seem to act solely on this enzyme since the protective action of the nucleoside against ethanol-induced fatty liver is due to a re-oxidation of reducing equivalents [7, 8], and, in this study, we have also evidenced an effect of adenosine on lipid peroxidation.

We are currently studying the possible mechanisms through which adenosine affects the CCl_4-induced lipoperoxidation and exerts its protective action against liver damage.

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