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MECHANISM OF THE FATTY LIVER INDUCED BY CYCLOHEXIMIDE AND ITS REVERSIBILITY BY ADENOSINE

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Abstract—The administration of cycloheximide to fasted rats produced a 2- to 3-fold increase in hepatic triacylglycerols. The antibiotic decreased the serum level of free fatty acids, triacylglycerols and ketone bodies. It also increased the incorporation of radioactive palmitate into liver lipids. It is suggested that an increased uptake of free fatty acids and an altered partition between oxidation and esterification are involved in the formation of fatty liver by this hepatotoxin. The simultaneous administration of adenosine prevented the formation of fatty liver. It is proposed that the effect of the nucleoside may be related to a decrease in the uptake of fatty acids by the liver.

In recent years there has been a great interest in using inhibitors of protein synthesis to investigate the pathogenesis of the fatty liver. The impairment of the biosynthesis of apolipoproteins and hence of their release has been considered the main factor in the formation of the fatty liver [1–3]. Jazcilevich and Villa-Treviño [4] have shown that the administration of cycloheximide (CHM), a powerful inhibitor of protein synthesis, to rats produces a 2- to 3-fold increase in the amount of triacylglycerols in the liver. This effect of the antibiotic has been confirmed both *in vivo* [5, 6] and *in vitro* [7]. However, Sabesin [8] has shown recently that acetoxycycloheximide, the most powerful inhibitor of protein synthesis in mammalian tissue, neither increased hepatic triacylglycerols nor diminished their concentration in plasma. This author concluded that hepatic inhibition of protein synthesis is not sufficient to produce an accumulation of triacylglycerols in the liver and suggested that in the pathogenesis of the fatty liver produced by CHM an increased mobilization of free fatty acids (FFA) may be involved [8]. Nevertheless, it has been shown previously that CHM does not increase the level of FFA in serum, but rather decreases it [5, 9]. This effect is due to an enhanced esterification of FFA in adipose tissue [9–11].

The present work was undertaken in an effort to get a clearer picture of the mechanism(s) involved in the genesis of the fatty liver produced by CHM. In addition, since it was shown previously that the administration of adenosine partially prevented the ethanol-induced fatty liver [12], the possibility that the nucleoside may protect against the accumulation of lipids in the liver produced by CHM was also tested.

MATERIAL AND METHODS

Cycloheximide, adenosine, 3-hydroxybutyrate dehydrogenase and zeolite were obtained from the Sigma Chemical Company (St. Louis, MO). Palmitic acid[1-^{14}C] as the sodium salt (17.8 mCi/m-mole) and DL-leucine[4,5-^{3}H], (50 mCi/m-mole) were purchased from the International Chemical & Nuclear Corp. (Irvine, CA). Coenzymes were obtained from Boehringer und Soehne (Mannheim). Other chemicals used were reagent grade of the best quality available.

The experiments were performed with male Wistar rats weighing between 140 and 180 g and fasted for 16–20 hr. The animals were injected intraperitoneally with saline (0.85% NaCl) or CHM (3.3 mg/kg of body weight) dissolved in saline and with saline or adenosine (200 mg/kg of body weight) suspended in saline. Therefore, each animal was injected twice, one injection immediately after the other. Four groups of animals were formed in each experiment, i.e. saline + saline, saline + adenosine, CHM + saline and CHM + adenosine. Other conditions used are indicated in the tables or in the text.

Hepatic triacylglycerols were determined by the method of Butler *et al.* [13]. Serum triacyglycerols and serum FFA were quantified using the methods of Van Handel and Zilversmit [14] and Dole and Meinertz [15] respectively. Blood ketone bodies were quantified enzymatically: 3-hydroxybutyrate (3-OHB) and acetoacetate (AA) according to Mellanby and Williamson [16] and Williamson and Mellanby [17] respectively. Total ketone bodies were calculated as the sum of 3-OHB plus AA.

To study hepatic protein synthesis, animals were injected with radioactive leucine (50 μCi/kg body weight) 30 min before being killed. The incorporation of the amino acid into liver proteins and the acid-soluble radioactivity were determined essentially as described by Jazcilevich and Villa-Treviño [4]. The incorporation of radioactive palmitate into liver and adipose tissue lipids was studied in pulse-type experiments in which the animals were injected intraperitoneally with palmitate (10 μCi/kg of body weight). After 15 min they were killed and samples of the liver and the epididymal fat pads were removed, weighed and homogenized. Lipids were extracted by the method of

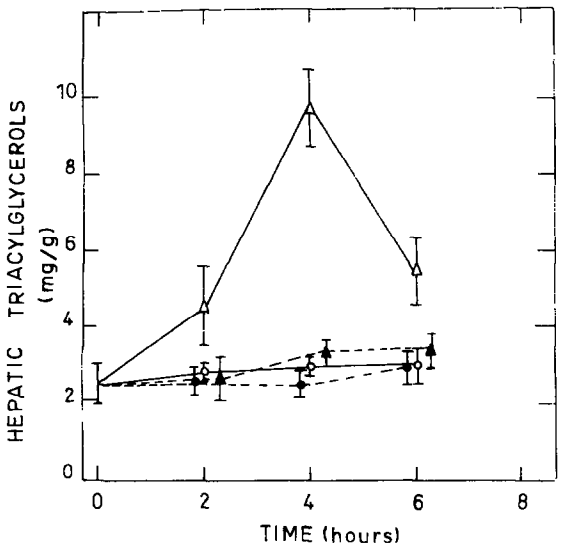

Fig. 1. Time course of the effect of cycloheximide and adenosine on hepatic triacylglycerols. Vertical lines represent the standard error of the mean of at least five determinations. Key: (○——○) saline + saline, (●– – –●) saline + adenosine, (○------○) cycloheximide + saline, and (▲– – –▲) cycloheximide + adenosine.

Folch *et al.* [18] and radioactivity in the lipid extracts was counted as described previously [9]. Statistical significance between comparable groups was determined by Student's *t*-test.

RESULTS

The administration of cycloheximide to fasted male rats increased the amount of triacyglycerols in the liver (Fig. 1). A maximal, 3-fold increase was detected 4 hr after treatment, following which the effect declined (Fig. 1). Adenosine alone produced only minor modifications in the amount of triacyglycerols in the liver but blocked completely the production of fatty liver by CHM (Fig. 1).

Table 1. Effect of cycloheximide and adenosine on the concentration of liver triacylglycerols in male and female rats*

Treatment	Liver triacylglycerols (mg/g) Males	Females
Saline + saline	2.99 ± 0.27 (5)	8.60 ± 1.30 (6)
Saline + adenosine	2.28 ± 0.38 (5)	5.12 ± 1.27 (7)
CHM + saline	9.74 ± 1.04† (5)	22.12 ± 1.91‡ (6)
CHM + adenosine	3.30 ± 0.41§ (5)	14.08 ± 1.08‖ (8)

* Determinations were made 4 hr after treatment. The results are expressed as the mean ± the standard error of the mean with the number of animals in parentheses.

† $P < 0.001$, compared to the saline + saline group (males).

‡ $P < 0.001$, compared to the saline + saline group (females).

§ $P < 0.001$, compared to the CHM + saline group (males).

‖ $P < 0.005$, compared to the CHM + saline group (females).

It is well known that female rats show increased susceptibility to fatty liver induction after treatment with hepatotoxins [19], so the effect of CHM in female rats was tested. It was observed that female rats had an increased amount of triacylglycerols in the liver, compared with males (Table 1). Cycloheximide produced a 2.5-fold increase in the amount of hepatic triacylglycerols in female rats and this effect was also blocked by adenosine (Table 1). In other words, the effects of CHM and adenosine on hepatic triacylglycerols were observed in both male and female rats.

The possibility that adenosine might reverse the inhibition of protein synthesis produced by CHM was considered and tested. As expected, CHM decreased the incorporation of leucine (≈95 per cent) into liver proteins and increased the amount of the label in the acid-soluble fraction (Table 2). Adenosine was unable to reverse this inhibition of protein synthesis and actually seemed to magnify it (Table 2).

The effects of CHM and adenosine on serum lipids were studied. Cycloheximide decreased the amount of triacylglycerols in the serum by 55 per cent and adenosine was unable to restore it to its normal value (Table 3). As has been reported previously, CHM produced a marked decrease (about 40 per cent) in the concentration of FFA in the serum (Table 3) [5, 9] which was not modified appreciably by the nucleoside.

The level of FFA in the serum results from a balance between lipolysis, mainly in adipose tissue, and utilization, mainly in adipose tissue and liver. The incorporation of radioactive palmitate into total lipids of adipose tissue and liver was studied in pulse-type experiments, as an index of the utilization of FFA. Cycloheximide produced marked increases in the incorporation of palmitate in both adipose tissue and liver (Table 4). Adenosine markedly decreased this effect of CHM on the liver but only slightly affected its action on adipose tissue, suggesting a different sensibility (Table 4).

Table 2. Effects of cycloheximide and adenosine on the incorporation of leucine into rat liver protein*

Treatment	Protein (cpm/mg protein)	Acid-soluble fraction (cpm/mg liver)
Saline + saline	327.04 ± 14.83 (5)	8.97 ± 0.28 (5)
Saline + adenosine	316.08 ± 10.76 (5)	10.41 ± 0.79 (5)
CHM + saline	39.42 ± 3.91† (5)	15.92 ± 0.28† (5)
CHM + adenosine	18.64 ± 1.40†,‡ (5)	15.92 ± 2.28§ (5)

* Indications as in Table 1.
† P < 0.001, compared to the saline + saline group.
‡ P < 0.005, compared to the CHM + saline group.
§ P < 0.02, compared to the saline + saline group.

The amount of ketone bodies in the blood reflects the oxidation of long-chain fatty acids by the liver [20]. Cycloheximide produced a strong decrease in total ketone bodies that resulted from a diminution in the levels of both 3-OHB and AA without modifying the 3-OHB/AA ratio (Table 5). Adenosine was not able to reverse this effect and no direct action of the nucleoside on this parameter was detected at the time tested (Table 5).

DISCUSSION

The present study confirms that the administration of CHM to rats produces fatty liver (Fig. 1, Table 1) [4–6]. As expected, the antibiotic markedly decreased the serum level of triacylglycerols (Table 3). It also decreased the serum level of FFA (Table 3), as has been shown previously [5, 9]. Hence, the hypothesis that an oversupply of FFA is involved in the pathogenesis of the fatty liver induced by CHM [8] may be discarded.

Table 3. Effects of cycloheximide and adenosine on serum lipids*

Treatment	FFA (μequiv/liter)	Triacylglycerols (mg/100 ml)
Saline + saline	480.78 ± 64.54 (8)	63.12 ± 8.96 (8)
Saline + adenosine	487.33 ± 49.76 (6)	65.94 ± 5.31 (4)
CHM + saline	304.38 ± 39.92† (6)	28.81 ± 3.15‡ (5)
CHM + adenosine	267.82 ± 33.16§ (6)	35.87 ± 4.74† (4)

* Indications as in Table 1.
† P < 0.05, compared to the saline + saline group.
‡ P < 0.01, compared to the saline + saline group.
§ P < 0.02, compared to the saline + saline group.

Table 4. Effects of cycloheximide and adenosine on the incorporation of palmitate into lipids of the liver and epididymal fat pads*

	Incorporation (cpm/mg wet wt)	
Treatment	Liver	Fat pads
Saline + saline	975.16 ± 168.62 (5)	1372.22 ± 227.48 (5)
Saline + adenosine	872.80 ± 170.96 (4)	2382.46 ± 532.12 (4)
CHM + saline	1901.20 ± 79.27† (4)	4570.05 ± 673.25† (4)
CHM + adenosine	920.50 ± 97.08‡ (4)	3269.60 ± 347.99† (4)

* Indications as in Table 1.
† P < 0.005, compared to the saline + saline group.
‡ P < 0.001, compared to the CHM + saline group.

Table 5. Effects of cycloheximide and adenosine on the blood level of ketone bodies*

Treatment	3-OHB	AA	Total ketone Bodies	3-OHB
	(μmoles/100 ml)			AA
Saline + saline	18.18 ± 1.70 (6)	21.00 ± 2.50 (6)	39.19 ± 2.96 (6)	0.87
Saline + adenosine	20.43 ± 3.05 (6)	21.86 ± 1.86 (6)	42.24 ± 2.89 (6)	0.93
CHM + saline	7.23 ± 1.49† (5)	7.53 ± 1.03† (5)	15.23 ± 2.02† (5)	0.96
CHM + adenosine	9.30 ± 1.30‡ (5)	9.14 ± 1.55‡ (5)	18.44 ± 1.86† (5)	1.02

* Indications as in Table 1.
† P 0.001, compared to the saline + saline group.
‡ P 0.005, compared to the saline + saline group.

The incorporation of radioactive palmitate was increased 2-fold in rats treated with CHM (Table 4). But, since the amount of FFA in the serum of these rats was decreased by 40 per cent (Table 3), the increased incorporation seems to be due to a minor isotopic dilution and therefore the net incorporation of FFA may have been the same. Nevertheless, it is well known that the uptake of FFA by the liver is directly proportional to the concentration to which it is exposed [21]. Since in rats treated with the antibiotic the serum level of FFA was lower, the results suggest that the uptake of FFA by the liver is increased probably due to the structural changes in cellular membranes of the hepatocyte, as reported previously [22]. However, the possibility that the increased incorporation of radioactive palmitate may have resulted from an accumulation of label due to the inhibition of protein synthesis cannot be ruled out completely in spite of the fact that the experiments were pulse-type. The increased incorporation of palmitate into epididymal fat pad lipids is in agreement with the stimulation of the esterification process produced by the antibiotic [9–11].

Cycloheximide decreased the blood level of ketone bodies by approximately 60 per cent (Table 5). This effect may be due to a diminished availability of fatty acids within the hepatocyte, which seems unlikely taking into account the points previously discussed, or to a change in the partition between esterification and oxidation. In addition, preliminary evidence suggests that CHM inhibits the oxidation of FFA. Liver homogenates from CHM-treated rats exhibit a diminished production of AA from exogenously supplied fatty acids.

In conclusion, the production of fatty liver by CHM seems to be due to an enhanced uptake of FFA (Tables 3 and 4) and an altered partition between oxidation and esterification (Tables 1 and 5) in the presence of a strong inhibition of protein synthesis (Table 2).

It was observed that female rats had a larger amount of triacylglycerols in the liver than males (Table 1). This difference has been observed previously by other authors [23] and it is probably related to the different metabolism of free fatty acids by male and female rat livers [24].

Adenosine completely prevented the formation of fatty liver by CHM (Fig. 1 and Table 1). The nucleoside affected neither the inhibition of protein synthesis nor the decrease in serum triacylglycerols produced by the antibiotic (Tables 2 and 3). Therefore, an increased output of fat from the liver is not the mechanism of adenosine action. The effect of CHM to decrease the serum level of FFA was not modified by the nucleoside, but the incorporation of radioactive palmitate into liver lipids was strongly diminished as compared with that of the CHM + saline group (Table 4). This result suggests that livers treated with CHM plus adenosine do not present the enhanced uptake of fatty acids produced by the antibiotic. This point is supported by the inhibition of acyl-CoA synthetase produced by adenosine both *in vivo* and *in vitro* [25]. The activity of this enzyme is essential for the hepatic metabolism of fatty acids supplied by the depots. The effect of adenosine on this enzyme is transient [25], and no action on FFA uptake was observed in rats treated with saline + adenosine (Tables 3 and 4). Therefore, in rats treated with CHM + adenosine, a lengthening of this action of adenosine seems to exist (Tables 3 and 4).

The diminution in the level of serum ketone bodies in rats treated with CHM + adenosine was similar to that observed in rats treated with CHM + saline. It is not clear yet if this action is due to a diminished uptake of FFA (Tables 3 and 4) or to the effect of CHM on FFA oxidation. Previously, it was reported that adenosine decreased the level of ketone bodies in serum [25], but the result observed in rats treated with CHM + adenosine (Table 5) does not seem to be related to this effect of the nucleoside, since adenosine profoundly affects the 3-OHB/AA ratio and in the present study no modification of this parameter was observed.

In a previous paper it was shown that adenosine partially blocked the ethanol-induced fatty liver [12]. This action was strongly related to the effects of ethanol and adenosine on the cytoplasmic redox state [12]. In this paper another mechanism by which adenosine prevents fatty liver is presented: a diminution of the hepatic uptake of fatty acids provided by the depots. Since the enzyme that regulate fatty acid metabolism are membrane associated and CHM has been shown to have an important action in the structure and function of cell membranes of the liver [22], the interaction of the antibiotic with the nucleoside in the induction of the fatty liver could possibly occur at membrane level.

Experiments are in progress to clarify some of the points raised in this discussion.

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