Inhibitory effect of D-galactosamine administration on fatty acid oxidation in rat hepatocytes

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1. INTRODUCTION

Important similarities between D-galactosamine (GaIN)-mediated hepatocellular necrosis and viral hepatitis have been emphasized [1]. The necrosis induced by GaIN is however accompanied by hepatic triacylglycerol (TAG) accumulation [2], an association not present in human viral hepatitis.

Koff et al. [3] first reported data suggesting that impaired hepatic lipoprotein release, presumably due to inhibition of synthesis of the protein moiety of very low density lipoproteins, is the mechanism of the GaIN-induced fatty liver. In further studies, in which a lower GaIN dose was used, Sabesin and Koff [4], observed, in the absence of any necrosis, the development of steatosis which was considered by the authors to result from an increased free fatty acid (FFA) influx rather than to an inhibition of TAG release. This hypothesis was further supported by Katterman and Sirowej [5] who reported that the plasma FFA level was increased 24 and 48 h after the intraperitoneal injection of GaIN. However, as no increase in plasma FFA was observed by these authors 12 h after GaIN administration, and since TAG accumulation had already occurred 4 h after GaIN administration, it seems highly unlikely that an increased FFA influx may play a major role in the development of the GaIN-induced fatty liver.

The purpose of the present study was to establish the factor(s) responsible for the TAG accumulation which is observed in rat liver as early as 3 h after a single dose of GaIN. Having confirmed that no increase in the serum FFA level is apparent at this stage of intoxication, this preliminary report was devoted to the study of the rate of fatty acid oxidation in isolated hepatocytes and mitochondria from GaIN treated rats.

The results show (i) that palmitate but not octanoate oxidation was markedly depressed in hepatocytes from GaIN treated rats, and (ii) that palmitoyl CoA but not palmitoyl L-carnitine oxidation in isolated mitochondria was affected. These data indicate therefore that GaIN administration impairs the rate of fatty acid oxidation in the liver, and suggest that this action results from a disturbance located at the carnitine palmitoyltransferase I level.

2. MATERIALS AND METHODS

Collagenase from *Clostridium histolyticum* (Type IV), D(+) galactosamine–HCl, palmitoyl L-carnitine-HCl, L-carnitine and bovine serum albumin (fatty acid free-fraction V) were purchased from Sigma Chemical Co., [1-^{14}C]octanoic acid sodium salt (spec. act. 30 mCi/mmol) and [U-^{14}C]-palmitic acid (spec. act. 403 mCi/mmol) from Amersham. All other reagents were of analytical grade or of the highest purity available (Sigma, Merck or Fluka).

Male Wistar rats (C.E.R.J., F-53680) weighing 200–240 g were kept at 20–22°C with a photoperiod of 12D–12N. They were starved overnight before the experiments and given water ad libitum.

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At 07:30 h D-galactosamine–HCl (neutralized at pH 7.4 with 2 N NaOH) was administered [278 μM (60 mg)/100 g body wt] intraperitoneally, control rats receiving an equal volume of saline.

Serum FFA were determined after Duncombe et al. [6] and aspartate aminotransferase (ASAT) (EC 2.6.1.1) activity according to Ceriotti [7] in blood collected after decapitation. Liver was quickly excised after decapitation and TAG determined by the method of Eggstein et al. [8].

For the preparation of isolated hepatocytes the animals were anesthetized by intraperitoneal injection of 50 mg sodium pentobarbital/100 g body wt and hepatocytes were isolated by collagenase perfusion [9] in a Krebs–Ringer bicarbonate buffer (pH 7.4), gassed with O_2 : CO_2 (95 : 5). Integrity of hepatocytes from both control and treated rats was routinely assessed by measuring lactate dehydrogenase release into the medium [10] and by cell membrane refractivity under phase-contrast microscopy. The viability of preparations from control and GaIN treated animals remained identical and was usually 94–96%. Suspensions were diluted in the same buffer supplemented with defatted albumin (final concentration: 1%) in order to obtain 5–6 × 10^6 cells/ml corresponding to 5–6 mg protein/ml.

For the determination of palmitate or octanoate oxidation, 2 ml aliquots of this hepatocyte suspension were incubated under O_2 : CO_2 (95 : 5) at 37°C in 25 ml Erlenmeyer flasks provided with center wells equipped with a folded filter (Whatman GF/A) impregnated with 0.1 ml 2 N KOH. The flasks were shaken in an incubator shaker (New Brunswick Scientific Co.) at 100 rev./min. After a preincubation period (10 min), 0.1 ml of the amino acid mixture suggested for energetic supply by Seglen [11] and labelled precursors (0.1 ml) were added to the hepatocyte suspension. These labelled precursors consisted of 0.5 μCi/1.1 μmol palmitate or octanoate. 30 min after the addition of precursors $^{14}CO_2$ production was determined according to Capuzzi et al. [12]. All assays were triplicated.

For the determination of the intracellular ^{14}C incorporation from labelled fatty acid, the hepatocyte suspension was incubated during 30 min. in nalgene Erlenmeyer flasks under the same experimental conditions. The reaction was stopped by refrigeration at 4°C and by adding 110 μmol non-labelled fatty acid as an albumin complex; samples were immediately centrifuged (500 × g for 2 min), hepatocytes in the pellets were rinsed twice in Krebs–Ringer buffer. The pellets were resuspended in a final volume of 1 ml and 0.1 ml aliquots were counted in scintillation vials containing 10 ml of scintillation 299 TM (Packard).

For the isolation of mitochondria, livers from treated and control animals were perfused with a saline solution at 4°C during 5 min. Liver mitochondria were prepared by the method of Beattie [13]. The fluffy layer was discarded, and the mitochondrial pellet washed three times in 0.25 M sucrose at 4°C. The pellet was finally resuspended in the same medium at a protein concentration of 50–75 mg/ml (protein was determined after [14]). Mitochondrial respiration was measured as previously described [15] at 25°C using a Gilson oxygraph equipped with a Clark oxygen electrode.

Statistical significance of results was assessed using Student's paired t-test.

3. RESULTS

As seen in table 1, a significant increase of liver TAG is apparent 3 h after administration of GaIN. At this time no modification in the serum ASAT activity is apparent; the serum FFA level is also unimpaired.

Hepatocytes isolated from rats at the same stage of GaIN intoxication and incubated with [^{14}C]palmitate show (table 2) a 52% inhibition in $^{14}CO_2$ production, inhibition which is not prevented when 1 mM L-carnitine is added to the medium. $^{14}CO_2$ production from [^{14}C]octanoate is unimpaired in the same conditions.

No disturbance in the intracellular incorporation of the label is apparent in hepatocytes isolated from GaIN treated rats incubated 30 min either with labelled palmitate [control: 3770 ± 272 cpm · 10^6 cells^{-1} (n = 5); treated: 3646 ± 353 (n = 5)] or labelled octanoate (data not shown).

Mitochondria from GaIN treated rats show no abnormalities in the respiration rate with palmitoyl L-carnitine as a substrate while oxidation rate of palmitoyl CoA is significantly decreased (–35%).

Table 1

Hepatic triacylglycerol level, serum free fatty acid concentration and aspartate aminotransferase activity 3 h after D-galactosamine administration

	Liver tag		Blood serum	
	μmol/g wt wt	μmol/liver	FFA mEq/l	ASAT U/l
Control	8.58 ± 0.42	49.13 ± 2.90	0.616 ± 0.025	87.2 ± 12.3
Treated	11.23 ± 0.22[a]	72.16 ± 4.17[a]	0.676 ± 0.050[b]	94.8 ± 10.3[b]

[a] $P < 0.001$; [b] $P > 0.05$

Experiments were performed as described in the text. Each value is the mean ± SEM of 8 expt

Table 2

Formation of $^{14}CO_2$ from [U-^{14}C]palmitate and [1-^{14}C]octanoate by hepatocytes from D-galactosamine treated rats

^{14}C-Labelled fatty acid		Control	Treated
Palmitate		231 ± 12	111 ± 17[a]
	+ 1mM L-carnitine	226 ± 11	112 ± 4[a]
Octanoate		115 ± 17	108 ± 25[b]

[a] $P < 0.001$; [b] $P > 0.05$

The cells were preincubated for 10 min in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 1% defatted albumin and incubated during 30 min in the presence of 0.5 mM [U-^{14}C]palmitate or 0.5 mM [1-^{14}C]octanoate. Production of $^{14}CO_2$ is expressed as cpm . 10^6 cells^{-1}. The results are mean values ± SEM of 4 to 6 triplicate experiments

Table 3

Effect of D-galactosamine administration on palmitoyl L-carnitine and palmitoyl-CoA oxidation

Substrates	Animals	O_2 consumption (nmol . min^{-1} . mg protein^{-1})	
		State 4	State 3
None	Control	9.0 ± 0.9 (5)	–
	Treated	10.7 ± 0.9 (5)	–
Palmitoyl L-carnitine (0.025 mM)	Control	15.6 ± 1.2 (6)	37.2 ± 2.7 (6)
	Treated	16.9 ± 1.7 (6)	41.5 ± 2.6 (6)
Palmitoyl-CoA (0.025 mM)	Control	12.1 ± 0.7 (3)	26.6 ± 0.6 (3)
+ L-carnitine (2 mM)	Treated	11.8 ± 1.2 (3)	17.4 ± 1.0 (3)[a]

[a] $P < 0.001$

Oxidation rate was measured by polarography in isolated mitochondria from fasted rats. The results are mean values ± SEM with the number of observations in parentheses

4. DISCUSSION

The present data show that the intraperitoneal administration of a single dose of 0.6 g GaIN per kg body wt results in an accumulation of liver TAG which is apparent at a stage where the absence of increase in the serum ASAT level suggests the absence of liver necrosis. At the same time, the serum FFA level is not increased suggesting that increased peripheral lipolysis resulting in enhanced blood FFA supply to the liver is not a prerequisite for fatty liver induction by GaIN.

The drastic decrease in palmitate oxidation to CO_2 found in hepatocytes isolated at this stage of intoxication suggests that an impairment in liver fatty acid oxidation may play an essential role in the induction of liver TAG accumulation following GaIN administration.

Contrary to the results concerning palmitate, no inhibition was found in the oxidation of octanoate. Therefore, decreased CO_2 production from palmitate in treated rats does not appear to be the result of endogenous dilution of the fatty acid pool.

Octanoate, a short-chain fatty acid which can enter the mitochondria without the intervention of carnitine acyltransferases [16]. This finding shows that the disturbance responsible for defective palmitate oxidation is localized at a level which precedes β-oxidation and which shows a specificity with respect to the fatty acid substrate, rather than at the β-oxidation level and/or the subsequent tricarboxylic acid cycle.

The results obtained when studying palmitoyl L-carnitine oxidation in mitochondria isolated from D-GaIN treated rats confirm the absence of disturbances in these mitochondrial processes. They suggest furthermore that the carnitine palmitoyltransferase II localized on the inner mitochondrial membrane is also unaffected. The total intracellular radioactivity found after incubation with [^{14}C]palmitate is unimpaired in hepatocytes isolated from such animals makes it unlikely that GaIN treatment affects primarily either palmitate uptake into the hepatocytes. Since palmitoyl-CoA oxidation is impaired by D-GaIN administration in isolated mitochondria, it is likely that GaIN acts mainly at the level of carnitine palmitoyltransferase I, which catalyses the conversion of palmitoyl-CoA to palmitoylcarnitine outside the mitochondrial permeability barrier for acylCoA. A decrease in the carnitine cell content is apparently not involved, as addition of carnitine to the medium does not prevent the inhibition of palmitate oxidation in hepatocytes from GaIN-treated rats. Further research is in progress to ascertain whether GaIN administration affects directly carnitine palmitoyltransferase I activity or acts on this enzyme through changes in the concentration of malonyl-CoA, the effector of the transferase [16].

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