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Biochemical Mode of Action of a Hepatoprotective Drug: Observations on (+)-Catechin

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RYLE, P. R., J. CHAKRABORTY AND A. D. THOMSON. *Biochemical mode of action of a hepatoprotective drug: Observations on (+)-catechin.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 473–478, 1983.—(+)-Catechin inhibits the hepatic lipid accumulation resulting from chronic ethanol ingestion in the rat. Experiments have been carried out to determine the mechanisms underlying this effect. Ethanol was administered (2.0 g/kg intraperitoneally) to Wistar rats and 90 min later 1 μCi [U-^{14}C] palmitic acid injected intraperitoneally. Animals were sacrificed 10 min after injection of palmitate and the liver freeze-clamped. Ethanol caused a 250% increase in the hepatic lactate:pyruvate (L:P) ratio and a 100% increase in the amount of [U-^{14}C] palmitate incorporated into the hepatic lipids when compared with controls. Pretreatment of animals with (+)-catechin (200 mg/kg orally) at 24 and 0 hr before ethanol caused significant reductions in the L:P ratio and amount of radioactivity incorporated into hepatic lipids, when compared with animals receiving ethanol alone. (+)-Catechin also stimulated $^{14}CO_2$ production from [1-^{14}C] palmitate by liver slices taken from rats 90 min after ethanol administration. Thus, (+)-catechin appears to mediate its effect on fat accumulation partly by correcting the ethanol-induced alterations in hepatic redox state as there is no evidence of the drug inhibiting ethanol metabolism.

Fatty liver Bioflavonoids Lipids Alcohol, ethyl

THE bioflavonoid compound (+)-catechin [(+)-cyanidanol-3] has been shown to exert a number of hepatoprotective effects in man and animals. Clinical studies have indicated that it lowers the serum titre of Hepatitis B surface antigen and improves serum markers of liver function in patients suffering from acute viral hepatitis [6,17]. The drug is a powerful free-radical scavenger and an antioxidant, protecting the rat liver endoplasmic reticulum against carbon tetrachloride toxicity *in vitro* [8]. It has also been shown to possess marked membrane-stabilizing properties *in vitro* [14].

Gajdos *et al.* [10] reported that (+)-catechin normalized the elevated hepatic NADH:NAD ratio and decreased ATP concentrations that are found during ethanol intoxication. These metabolic disturbances produce a fatty liver by decreasing fatty acid oxidation and favoring incorporation of fatty acids into triglycerides. We have previously reported that simultaneous administration of (+)-catechin to rats during ingestion of a liquid diet containing 28% of the total calories as ethanol prevents the mild degree of fat accumulation induced by such a diet [16]. In the present study, a severe degree of fatty liver has been induced in rats by administering a liquid diet containing 36% of the total calories as ethanol, and the effect of simultaneous treatment of the animals with (+)-catechin was determined. In addition, some experiments have been carried out to determine the effect of the compound on hepatic lipid disposition following acute administration of ethanol, so as to assess whether there is any relationship between the drug's ability to correct the ethanol-induced disturbances in the hepatic redox state and increased turnover of lipids within the liver.

METHOD

Animals

Male Wistar rats (University of Surrey strain, 180–210 g) were used throughout. They were housed individually in wire-bottomed cages in a room maintained at 22°C and lighting was provided between 0700 and 2100 hours.

Chronic Fatty Liver Induction Study

Animals were randomly allocated into three groups (8 each) as follows: Group A: Controls; Group B: Liquid diet containing ethanol; Group C: Liquid diet containing ethanol—animals simultaneously given (+)-catechin.

The liquid diet used was as described previously [16] except that the final amount of ethanol in the diet was adjusted to give 36% of the total calories, replacing an isocaloric amount of glucose. On Days 1–7 of the study, all groups

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TABLE 1

EFFECT OF (+)-CATECHIN ON FATTY LIVER INDUCED BY CHRONIC ETHANOL CONSUMPTION IN THE RAT

	Group A Controls		Group B Ethanol		Group C Ethanol and (+)-Catechin
Total hepatic lipid (mg/g)	39.0 ± 8.9	$p<0.001$	153.1 ± 17.0	$p<0.001$	99.0 ± 16.3
Hepatic triglyceride (mg/g)	6.4 ± 2.1	$p<0.001$	59.0 ± 9.7	$p<0.001$	34.5 ± 7.4
Serum glutamate dehydrogenase activity (U/l)	5.1 ± 1.3	$p<0.001$	15.8 ± 6.9	$p<0.001$	6.6 ± 1.6

Three groups of 8 male Wistar rats were fed a liquid diet containing 36% of the total calories as ethanol over a period of 4 weeks. The pair-fed control group (A) received an isocaloric amount of glucose in place of ethanol. (+)-Catechin was given once daily to the animals in group C (200 mg/kg/day orally). Parameters shown were measured at the conclusion of the study. Results are given as means ± S.D.

received liquid diet containing no ethanol, then between Days 8 and 15, Groups B and C received liquid diet containing 20% of the total calories from ethanol while Group A received a liquid diet containing no ethanol. For the rest of the study (Days 16–36) the control group (A) continued to receive liquid diet without ethanol while Groups B and C were given liquid diet containing 36% of the total calories from ethanol. The daily dietary intake of the animals in Groups B and C was monitored and used to pair-feed the animals in Group A.

(+)-Catechin was given once daily (Days 1–36) as a suspension in water to the animals in Group C by gastric tube (200 mg/kg/day). The animals in Groups A and B received a similar volume of water daily by gastric tube.

On Day 36, the animals were anaesthetised with diethyl ether and blood was collected by closed cardiac puncture. The animals were then sacrificed by cervical dislocation and the livers removed for analysis. Determination of serum glutamate dehydrogenase activity and hepatic total lipid and triglyceride concentrations was carried out as described previously [16]. Histological examination of liver tissue was carried out on sections cut from frozen tissue, the lipid content being visualised by Oil Red "O" staining.

Hepatic Lipid Disposal and Turnover Studies

Animals were maintained on a standard laboratory chow and water diet and fasted for 48 hours prior to the experiment. [1-^{14}C] Palmitic acid (specific activity 49.2 Ci/mol), [U-^{14}C] palmitic acid (specific activity 403 Ci/mol) and ^{14}C-hexadecane were supplied by Amersham International, Amersham, Bucks, U.K.

Hepatic uptake of [U-^{14}C] palmitic acid was determined by the method of Abrams and Cooper [1] as modified by Beauge *et al.* [5]. Three groups of five animals were injected intraperitoneally with [U-^{14}C] palmitic acid (1 μCi/100 g body wt.) in freshly prepared rat serum, 90 min after a single administration of ethanol (2 g/kg intraperitoneally). Ten minutes later, the animals were sacrificed by cervical dislocation, the abdomen opened rapidly and a portion of the liver freeze clamped *in situ* with aluminium tongs which had been pre-cooled in liquid nitrogen. Hepatic tissue concentrations of lactate, pyruvate and α-glycerophosphate were determined in perchloric acid extracts of frozen tissue by the methods of Gutmann and Wahlefelt [11], Czok and Lamprecht [7] and Michal and Lang [13], respectively. The incorporation of [U-^{14}C] palmitic acid into hepatic lipids was determined after extraction from the tissue [9]. An aliquot of the final extract was counted for radioactivity incorporated into total lipids, while further aliquots were applied to a Silica Gel 60 thin layer chromatography plate alongside appropriate markers. The plate was developed in hexane:diethyl ether: acetic acid (70:30.1) and the spots visualized by placing the plate in a tank containing iodine vapor. The spots corresponding to triglycerides and phospholipids were scraped off the plate, extracted with chloroform:methanol (2:1) and counted.

Fatty acid oxidation rate was determined by measuring $^{14}CO_2$ production from [1^{14}-C] palmitate by liver slices [15]. Animals were sacrificed 90 min after a single administration of ethanol (2 g/kg IP), a portion of liver rapidly freeze clamped for metabolite determinations, the remainder then being used to prepare liver slices which were incubated for 90 min at 37°C in Krebs-Ringer phosphate buffer containing albumin-bound [1-^{14}C] palmitate. The incubation was stopped by the addition of trichloroacetic acid to the medium and the $^{14}CO_2$ was collected into Hyamine 10X and counted. Liver protein was determined according to Lowry [12]. All ^{14}C counting was done on a Nuclear Enterprises NE 8312 radioactivity counter. The counting efficiencies estimated by

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FIG. 1. Representative liver histology sections at the conclusion of the fatty liver study (×168: stain=Oil Red "O"). Top: controls; middle: ethanol; bottom: ethanol and (+)-catechin.

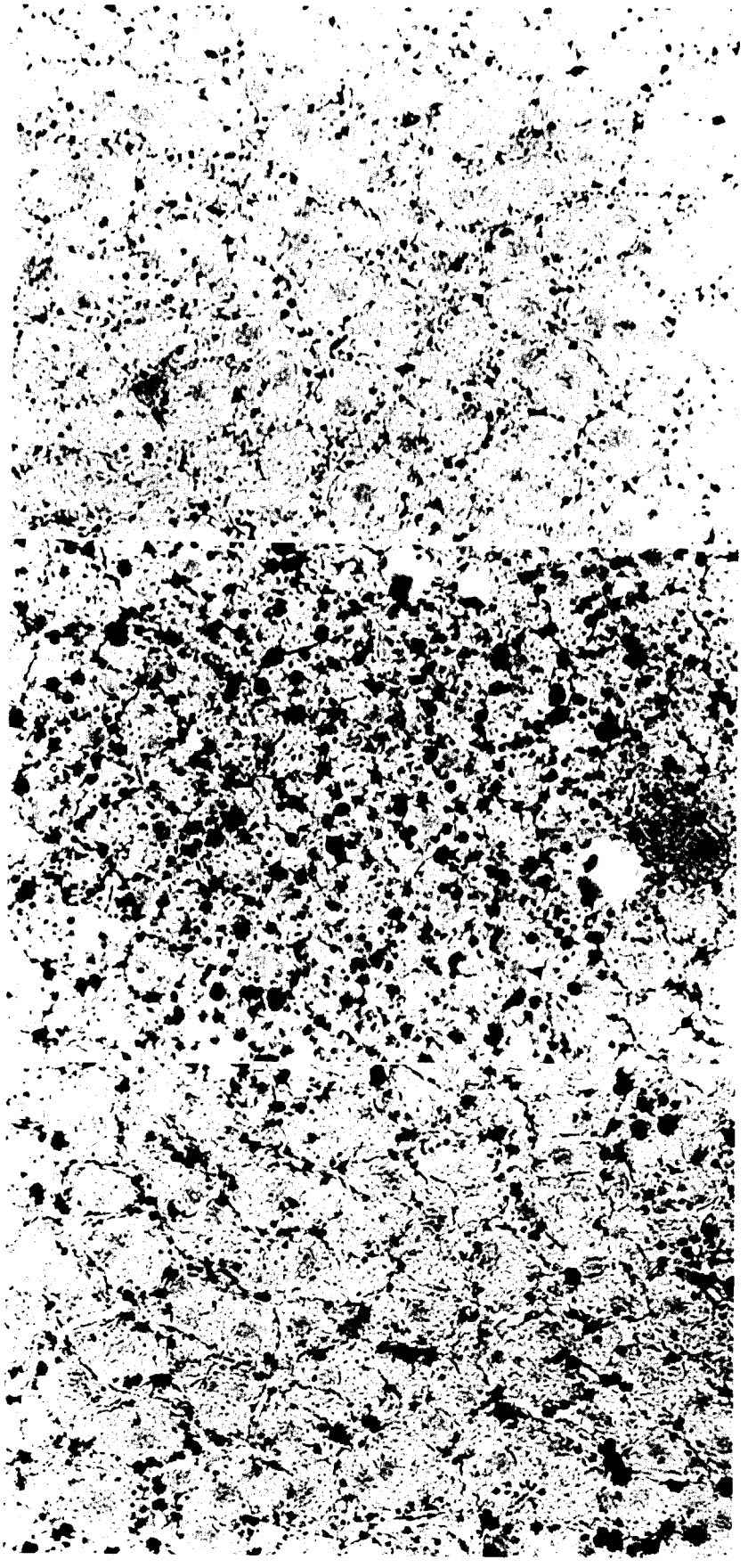

TABLE 2

EFFECT OF (+)-CATECHIN ON LIPID DISPOSAL IN RAT LIVER FOLLOWING AN ACUTE DOSE OF ETHANOL

	Treatment				
	Controls		Ethanol		Ethanol and (+)-Catechin
% injected [U-^{14}C] Palmitate in liver lipids	8.6 ± 1.2	$p<0.001$	16.7 ± 1.6	$p<0.001$	10.4 ± 1.9
Radioactivity incorporated into triglycerides (dpm/g liver)	9662 ± 1855	$p<0.001$	39393 ± 2999	$p<0.001$	18003 ± 2990
Radioactivity incorporated into phospholipids (dpm/g liver)	16063 ± 2562	$p<0.01$	24849 ± 4868	$p<0.001$	13870 ± 3965
Lactate (nmol/g liver)	1080 ± 143	$p<0.001$	2262 ± 174	$p<0.02$	1603 ± 305
Pyruvate (nmol/g liver)	168 ± 10	$p<0.001$	109 ± 10	$p<0.001$	169 ± 11
Lactate:Pyruvate	6.5 ± 1.2	$p<0.001$	20.8 ± 1.6	$p<0.001$	9.5 ± 1.9
α-glycerophosphate (nmol/g liver)	143 ± 10	$p<0.001$	412 ± 91	N.S.	366 ± 79
Serum ethanol (mg/100 ml)	0	—	230 ± 19	N.S.	210 ± 12

Rats (5 per treatment group) were given an intraperitoneal dose of [U-^{14}C] palmitic acid (1 μCi/100 g body weight) 90 min after a single dose of ethanol (2 g/kg IP). Ten minutes after injection of the palmitate the animals were sacrificed and the liver freeze-clamped rapidly. Control animals received water IP in lieu of ethanol. (+)-Catechin was given by gastric tube (200 mg/kg on each occasion) at 24 and 0 hrs before ethanol. Expression of results as in Table 1. N.S.=Not significant.

using ^{14}C-hexadecane as an internal standard were greater than 80%.

Ethanol determinations were carried out enzymatically on serum prepared from blood taken from the neck of the animals immediately after sacrifice.

Results are shown as means±SD. Statistical significance was determined using Student's t-test for paired data.

RESULTS

Hepatic total lipid and triglyceride concentrations and serum glutamate dehydrogenase (GLDH) activities at the conclusion of this study are shown in Table 1. Administration of (+)-catechin caused a significant reduction in the extent of fatty infiltration induced by chronic ethanol consumption in the animals, the biochemical findings being confirmed by histological examination of the tissue, which revealed reduced number of lipid globules and smaller areas of affected tissue in the drug treated animals when compared with animals given ethanol alone (Fig. 1). Administration of (+)-catechin did not cause any reduction in food intake when compared with animals given ethanol alone, and random serum ethanol determinations carried out during the study were comparable between the ethanol-treated groups, whether they received the drug or not. Thus, the effects of (+)-catechin cannot be attributed to reduced ethanol intake or altered ethanol metabolism.

Results from the hepatic lipid disposal and turnover studies are shown in Tables 2 and 3. (+)-Catechin significantly reduced the amount of labelled palmitate incorporated into hepatic lipids, particularly the triglyceride and phospholipid fractions, in ethanol-treated animals, these decreases being associated with normalization of the hepatic lactate:pyruvate ratio. The latter was mainly due to increased pyruvate concentrations. Similarly, (+)-catechin reversed the ethanol-induced inhibition of fatty acid oxidation by liver slices, this normalization also being associated with correction of the hepatic redox state, measured as the lactate:pyruvate ratio. In both the hepatic palmitate uptake and fatty acid oxidation experiments, (+)-catechin did not significantly reduce the large increase seen in hepatic α-glycerophosphate levels following ethanol administration. Serum ethanol estimations in these acute experiments indicated that (+)-catechin did not affect the degree of intoxication or elimination rate of ethanol.

DISCUSSION

Several factors are thought to contribute to the development of fatty liver. Disturbance of the hepatic NADH:NAD ratio due to ethanol oxidation leads to several metabolic alterations including decreased fatty acid oxidation, increased α-glycerophosphate formation from dihydroxyacetone phosphate, decreased Kreb's Cycle activity and possibly also increased fatty acid synthesis. These factors tend to favor disposal of fatty acids into triglycerides producing the characteristic alcoholic fatty liver. Disturbance of the hepatic redox state is one of the major causative factors in the development of chronically induced alcoholic fatty liver, and administration of substances that normalize the redox state during chronic ethanol consumption, for example, nicotinic acid, reduce the degree of fatty change [3]. Our acute experiments have clearly demonstrated that (+)-catechin is effective at reversing the redox state disturbance following ethanol administration, and this correction appears to correlate with reduced disposal of fatty acids into hepatic triglycerides and increased fatty acid oxidation rates. The time point chosen after ethanol administration in the acute experiments is optimal in terms of observing redox state disturbances, but unfortunately no significant differences in hepatic triglyceride concentrations were observable at this early stage of intoxication. The peak hepatic triglyceride concentration after acute ethanol administration is not reached until

TABLE 3

EFFECT OF (+)-CATECHIN ON FATTY ACID OXIDATION BY LIVER SLICES FROM ETHANOL PRETREATED RATS

	Treatment				
	Controls		Ethanol		Ethanol and (+)-Catechin
$^{14}CO_2$ released from liver slices in incubation (dpm/100 mg protein)	26506 ± 3920	$p<0.001$	9835 ± 1304	$p<0.001$	19671 ± 4049
Lactate (nmol/g liver)	918 ± 76	$p<0.001$	1748 ± 70	$p<0.001$	1323 ± 86
Pyruvate (nmol/g liver)	167 ± 13	$p<0.001$	112 ± 10	$p<0.001$	157 ± 14
Lactate:Pyruvate	5.7 ± 0.5	$p<0.001$	15.7 ± 1.3	$p<0.001$	8.6 ± 1.2
α-glycerophosphate (nmol/g liver)	142 ± 14	$p<0.001$	486 ± 83	N.S.	404 ± 44
Serum ethanol (mg/100 ml)	0	—	213 ± 29	N.S.	192 ± 24

Rats (5 per treatment group) were sacrificed 90 min after a single dose of ethanol (2 g/kg IP), a portion of liver freeze-clamped rapidly for metabolite determinations and the remainder used to prepare liver slices which were then incubated for 90 min in Krebs-Ringer phosphate buffer containing 1 μCi albumin-bound [1-^{14}C] palmitic acid and the $^{14}CO_2$ released was collected. Control animals received water IP in lieu of ethanol, and (+)-catechin was given by gastric tube (200 mg/kg on each occasion) at 24 and 0 hrs before ethanol. Expression of results as in Table 1.

16 hours or more after administration, when all the ethanol has been metabolized. Thus, care must be taken when relating acute effects of either ethanol or (+)-catechin to the development of chronic fatty liver. Acute administration of ethanol in large doses induces various factors which predispose to fatty liver formation. These include catecholamine-mediated mobilization of peripheral fatty acids, increased hepatic blood flow and corticosteroid-mediated induction of phosphotidate phosphohydrolase, the final enzyme in the pathway of triacylglycerol synthesis [2,4]. These acute effects of ethanol tend to mask other effects which may be of more importance in the development of fatty liver in the chronic situation.

Thus, (+)-catechin is able to reverse the acutely-induced disturbance in hepatic redox state following ethanol administration and reduce disposal of a peripherally-administered radiolabelled fatty acid into hepatic triglycerides. This action of the drug probably accounts, in part, for its ability to reduce hepatic fatty infiltration during chronic ethanol consumption. The exact mechanism by which (+)-catechin normalizes the redox state is not clear. Serum ethanol determinations have repeatedly shown that the compound does not affect uptake or elimination of ethanol. Gajdos *et al.* [10] suggested that (+)-catechin might stimulate glycolysis, thereby generating pyruvate to reoxidase NADH to NAD^+. If this was the case, however, one would expect to find elevated lactate concentrations in the liver when (+)-catechin was given with ethanol, and this was not so in our acute experiments. Therefore, the compound must stimulate NADH reoxidation by some other mechanism, possibly through activation of the mitochondrial respiratory chain.

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