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LIVER NADP AND $NADPH_2$ IN LIVER NECROSIS INDUCED BY CARBON TETRACHLORIDE: THE MODIFYING ACTION OF PROTECTIVE AGENTS

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Abstract—An investigation has been made of a previous speculation that the decreased content of NADP+$NADPH_2$ in rat liver 1 hr after the administration of carbon tetrachloride is associated with the sequence of events that progress to necrosis. A variety of agents have been administered together with the carbon tetrachloride to produce modifications in the extent of centrilobular necrosis present 24 hr later. The effects of such treatments on the level of NADP+$NADPH_2$ in the liver 1 hr after dosing have been determined.

It has been found that substances (Phenergan; Cetab; Nupercaine) that substantially delay the appearance of centrilobular necrosis (assessed histologically 24 hr after dosing) also prevent the drop in liver NADP+$NADPH_2$ that normally occurs 1 hr after administering carbon tetrachloride. Several agents (Benadryl; Anthisan; SKF-525A) that were partially protective in terms of delaying necrosis also prevented the nucleotide change. On the other hand, several agents (dodecyl sulphate, desferral, and propyl gallate) did not inhibit either the appearance of necrosis or the decreased nucleotide content.

Two substances, phenobarbitone and cysteamine, were found to have no significant effect on the development of necrosis yet prevented the nucleotide decrease. It is argued that the explanation of this effect lies in the transient nature of the protective action possessed by these materials.

The evidence obtained in this study is consistent with the previous speculation that the nucleotide decrease and the developments of necrosis are related in this type of liver injury.

A SINGLE dose of CCl_4 administered to rats results in severe centrilobular necrosis and in fatty degeneration of the liver.[1] Although these histological appearances do not become apparent until several hr after oral dosing, several biochemical changes are known to occur in the first hr of intoxication, particularly in components associated with the endoplasmic reticulum.[2–4] One early change, which appears of considerable importance in regard to the synthetic ability of the liver parenchyma, is a decrease of approximately 30 per cent in the sum of NADP + $NADPH_2$ that occurs in the liver during the first hr of intoxication.[5]

Many procedures are known that will delay or prevent the appearance of necrosis following the administration of CCl_4 to the rat.[6–8] In many instances these "protective agents" have little influence on the accumulation of fat in the liver and this apparent dissociation of the two most obvious features of liver damage following the administration of CCl_4 has led to the concept that the metabolic pathways leading to necrosis

on the one hand, or to fat accumulation on the other, are different at least in their terminal regions.[7]

In attempts to unravel the mechanism resulting in the decreased liver concentration of NADP + $NADPH_2$ following dosing with CCl_4 the question arose: is this decrease associated with the pathways leading on to necrosis or is the drop in $NADPH_2$ primarily involved in the processes producing a fatty liver? This paper presents work concerned with that question.

METHODS

The rats used were adult albino females, body wt. approx. 130 g; they were killed by cervical dislocation and pieces of liver were immediately removed for the assay of NADP and $NADPH_2$ by the method of Slater *et al.*[9]

Carbon tetrachloride was given orally under light ether anaesthesia as a 1 : 3 mixture in liquid paraffin (0·5 ml mixture was given/100 g body wt.). Controls received the equivalent volume of liquid paraffin. For experiments involving NADP and $NADPH_2$ estimations the rats were killed 1 hr after oral dosing. NADP and $NADPH_2$ estimations were always done on control and CCl_4-treated rats in each particular group on the same day so that direct comparison of the effects of drug treatment could be made. For histological assessment, groups of rats were killed 24 hr after oral dosing and liver sections were fixed in formol–saline and stained with haematoxylin–eosin. Histological examination of rats treated (a) orally with liquid paraffin and intraperitoneally (i.p.) with the drug and (b) orally with the CCl_4–liquid paraffin mixture and with the drug/i.p., was carried out for the following groups in Table 1: cysteamine; SKF-525A; sodium salicylate; propyl gallate plus vitamin C, and desferral. By visual assessment, the extent of necrosis in (a) and (b) was graded +, ++ or ++++ compared to rats that had received the CCl_4-liquid paraffin mixture alone (graded ++++). Histological examination of sections from (a) was performed to check upon the possibility that the drugs which were used themselves produced liver damage. The remaining groups in Table 1 have previously been classified in a similar manner.[10]

All "protective" and other drugs* were given i.p. under light ether anaesthesia. In most cases the injection was performed immediately prior to dosing with CCl_4; in some cases, however, pre-treatment of the rat with the drug was carried out. Details of the times of administration and dosage are given in Table 1. "Desferral" (desferrioxamine) was a generous gift from CIBA, Horsham, Ltd.; SKF-525A was kindly provided by Smith, Kline and French, Ltd.; Welwyn Garden City.

RESULTS

Table 1 shows the results obtained in this investigation. It can be seen that substances that were effective in substantially reducing the extent of liver necrosis, as assessed 24 hr after administration, were also effective in preventing the drop in NADP + $NADPH_2$ that normally occurs 1 hr after CCl_4-administration. Substances in this group were Cetab, Nupercaine, and Phenergan; in none of these cases was there any significant difference between the mean values for the sum NADP + $NADPH_2$ in the (control + drug) and the (CCl_4 + drug) groups.

* Abbreviations: Cetab—cetyltrimethylammonium bromide;
Phenergan—promethazine; Anthisan—mepyramine;
Benadryl—diphenhydramine.

TABLE 1. THE EFFECTS OF VARIOUS TREATMENTS ON THE LEVELS OF NADP AND $NADPH_2$ IN RAT LIVER, FOLLOWING DOSING WITH EITHER LIQUID PARAFFIN (LP) OR A CCl_4–LIQUID PARAFFIN MIXTURE

Agent	Dose (mg./100 g body wt.)	Time of dosing (min)	CCl_4-liquid paraffin or liquid paraffin at zero time	No. of rats	$\frac{\text{Liver wt.}}{\text{Body wt. (g)}} \times 100$	NADP+$NADPH_2$	P	$\frac{NADPH_2}{NADP}$	Necrosis
1. —	—	—	LP*	10	4·88 ± 0·36	1173 ± 87	<0·05	10·6 ± 0·73	++++
			CCl_4*	12	4·60 ± 0·21	855 ± 41		6·2 ± 0·70	
2. Cetab	0·5	0	LP	3	5·26 ± 0·67	1013 ± 81	N.S.	6·9 ± 0·51	+
			CCl_4	4	5·51 ± 0·51	942 ± 21		8·4 ± 1·07	
3. Nupercaine	1·44	−15	LP	3	4·95 ± 0·17	1273 ± 70	N.S.	7·0 ± 0·40	+
			CCl_4	3	4·47 ± 0·03	1146 ± 31		6·9 ± 0·27	
4. Cysteamine	3·4	−10	LP	2	4·70	998	—	6·5	++++
			CCl_4	4	4·47 ± 0·19	1027 ± 8		6·0 ± 0·71	
5. SKF-525A	4·0	−60	LP	5	4·57 ± 0·17	926 ± 71	N.S.	6·5 ± 0·78	++
			CCl_4	4	5·13 ± 0·15	826 ± 73		5·4 ± 0·79	
6. Salicylate	40·0	−60	LP	6	4·63 ± 0·33	751 ± 43	N.S.	5·1 ± 0·65	++++
			CCl_4	6	5·60 ± 0·47	689 ± 78		4·4 ± 0·61	
7. Anthisan	1·5	−15	LP	5	4·81 ± 0·46	1000 ± 56	N.S.	7·8 ± 0·79	++
			CCl_4	3	4·65 ± 0·18	990 ± 9		6·6 ± 0·43	
8. Benadryl	2·5	−15	LP	5	4·84 ± 0·25	1032 ± 68	N.S.	8·4 ± 0·78	++
			CCl_4	4	5·11 ± 0·29	885 ± 102		5·7 ± 0·48	
9. Phenobarbitone	2·4	−10	LP	4	4·23 ± 0·53	887 ± 96	N.S.	7·8 ± 1·43	++++
			CCl_4	8	4·60 ± 0·16	898 ± 68		6·7 ± 0·67	
10. Phenergan	2·5	0	LP*	4	4·90 ± 0·33	1144 ± 49	N.S.	8·2 ± 1·1	+
	1·25	5	CCl_4*	6	5·05 ± 0·35	1127 ± 56		12·0 ± 0·90	
11. Propyl gallate + vitamin C	30·0 + 80·0	0	LP	2	4·57	1075		9·5	++++
			CCl_4	2	4·66	645	—	5·0	
12. Dodecyl sulphate	2·5	0	LP	4	5·83 ± 0·37	1144 ± 53	0·02	10·1 ± 1·35	++++
			CCl_4	5	5·14 ± 0·30	898 ± 60		6·3 ± 1·05	
13. Desferral	10·0	−30	LP	4	4·60 ± 0·17	1027 ± 77	<0·05	11·0 ± 0·93	++++
			CCl_4	4	5·45 ± 0·42	768 ± 46		6·8 ± 1·11	

Nucleotide values are given as μg/whole liver/100 g body wt. (see ref. 5) and may be converted to μg/g wet wt. liver by dividing by column 6 (liver wt./100 g body wt.). Mean values ±S.E.M. are given. In all cases the rats were killed 1 hr after dosing with the liquid paraffin or the CCl_4-liquid paraffin mixture. The nucleotide results in groups 1 and 10, marked * are taken from ref. 5. Histological assessments for groups 1–3, 7–10 12 are taken from ref. 9. For other details see Methods.

Mean values for each group were compared by Student's t-test and the values of P are entered in column 7; N.S. signified that $P > 0·10$.

Substances that proved partially effective in reducing the extent of central necrosis under the experimental conditions used also tended to stop the decrease in the sum NADP + $NADPH_2$ found with CCl_4 alone. In this group were SKF-525A, Anthisan, and Benadryl. Several substances that did not affect the development of central necrosis did not affect the drop in the sum NADP + $NADPH_2$ produced by CCl_4 treatment alone; these substances were Desferral, dodecyl sulphate, and propyl gallate + vitamin C. Phenobarbitone and cysteamine showed a different behaviour in that they maintained the level of the sum NADP + $NADPH_2$ 1 hr after administering CCl_4 compared to the liquid paraffin treated control group, but had no effect on the extent of central necrosis assessed histologically some 24 hr later.

It can also be seen in Table 1 that administration of sodium salicylate alone produced a substantial decrease in the sum of NADP + $NADPH_2$ in the liver 1 hr after dosing and yet did not result in the appearance of significant centrilobular necrosis. The mechanism of the effect of sodium salicylate on nucleotide levels has been considered elsewhere.[11] Because of the effect of salicylate on the sum of NADP+ $NADPH_2$ in the liver, it is not possible to interpret unequivocally the action (if any) of that agent on the nucleotide drop produced by carbon tetrachloride.

DISCUSSION

Since "Phenergan" inhibits the rapid decrease in the sum of NADP + $NADPH_2$ in the liver[5] and delays the onset of centrilobular necrosis following the administration of carbon tetrachloride,[12] whilst having little effect on the accumulation of fat in the liver, the speculation has been made that the nucleotide change is associated with the necrogenic chain of reactions.[5] Of course, this does not mean that the decrease in the sum NADP + $NADPH_2$ by itself leads to necrosis, but simply that in the case of carbon tetrachloride-induced necrosis the nucleotide decrease observed is a contributory factor.

In an attempt to obtain evidence for the above speculation, twelve substances have each been administered concomitantly with carbon tetrachloride and the resultant effects on the liver nucleotide contents and the extents of centrilobular necrosis have been compared to the changes normally found after giving carbon tetrachloride itself. If the speculation mentioned above were true then correspondence should be observed between protection against the drop in NADP + $NADPH_2$ and protection against liver necrosis. Evidence for such a correlation has in fact been found in this study since all of the agents tested (Phenergan, Cetab, Nupercaine) that were protective against the onset of liver necrosis assessed histologically 24 hr after poisoning[10] also prevented the decrease in liver NADP + $NADPH_2$ produced 1 hr after giving carbon tetrachloride alone.

Further, no agent has been found in the course of this study that delays the appearance of centrilobular necrosis produced by CCl_4 (as assessed 24 hr after dosing) yet has no effect on the associated decrease in NADP + $NADPH_2$. Thus by these observations, the drop in NADP + $NADPH_2$ and the onset of centrilobular necrosis appear related.

Extending the above agument, it might be thought that the converse of the original speculation should also apply, namely: substances that have no effect in delaying the onset of centrilobular necrosis (assessed at 24 hr after dosing) should have no effect

in preventing the decrease in NADP + $NADPH_2$ measured 1 hr after CCl_4 administration. But for this to be generally true assumes a tighter relationship between the two events than might in fact exist, and exceptions to the converse statement given above are not necessarily inconsistent with the original speculation. The time-scales for the development of the two events studied are very different and it is possible that the early (nucleotide) effect may be temporarily delayed for an hour or so by a particular agent whereas such a transient effect on the development of the later event (necrosis) may not be readily detectable by the relatively crude histological procedures used.

In fact, although several agents (propyl gallate, dodecyl sulphate and desferral) satisfy the converse statement in that they have no effect on either event studied, two agents (cysteamine and phenobarbitone) had no appreciable effect in delaying the onset of necrosis yet maintained the sum of NADP + $NADPH_2$ at the control level. It is probable that these latter two exceptions are a result of transient early protective roles that quickly diminish with time due, for example, to rapid elimination of the agents from the body as a whole, or by metabolism of the agent to an inactive form in the liver itself, or by the agent being only transiently effective in preventing the build up of free radical chain reactions (see below).

Following a single intraperitoneal dose, cysteamine quickly accumulates in the blood and tissues but is also very rapidly metabolised and excreted.[13] The primary phase in acute liver injury induced by CCl_4 appears to involve an activation stage in which CCl_4 is metabolised to a free radical intermediate perhaps CCl_3. (for discussion of this point see refs. 4 and 14). Thus protection against CCl_4-induced liver injury may be obtained with free radical trapping agent such as cysteamine which, whilst present in adequate amounts, may be partially or fully effective in preventing serious liver derangements. Due to the rapid elimination of cysteamine, however, such protection is likely to be transient as, in fact, found here where the extent of centrilobular necrosis present 24 hr after poisoning was not appreciably diminished. Delays of a few hours in the initial built-up of the necrogenic chain of reactions would probably not be apparent with the procedure used here of histological examination at a single time (24 hr) when necrosis is normally well developed.

A similar argument based upon transient effects can be offered in explanation of the behaviour observed with phenobarbitone. For instance, it is possible that this agent exerts its "protective" role on the sum of NADP + $NADPH_2$ through a transient inhibition of peroxidative reactions sparked-off in the endoplasmic reticulum by CCl_4. The microsomal lipid peroxidation sequence has been suggested to be the major system involved in the primary activation stage by which CCl_4 is converted to CCl_3.[4] Also it is known that microsomal lipid peroxidation, coupled to the oxidation of $NADPH_2$, is inhibited by drugs undergoing oxidative metabolism in the endoplasmic reticulum.[16] Thus, by prolonging the induction period of peroxidative reactions involving $CCl_3^{\bullet}$, phenobarbitone can be seen to possess potential protective ability against liver damage induced by CCl_4. If this explanation is correct, then the delay in the development of necrosis produced by phenobarbitone is not long enough to be readily detectable at the 24 hr assessment period. Phenergan, on the other hand, produces quite a lengthy delay in the onset of necrosis: after the treatment used here necrosis is not evident at 24 hr but is fully developed 48 hr after the initial administration of CCl_4.[12] It is of interest that Phenergan is much more inhibitory than

phenobarbitone on the microsomal enzyme system coupling $NADPH_2$ oxidation, oxygen uptake and lipid peroxidation: Phenergan inhibits 50 per cent at 30 μM,[17] even with microsomal suspensions aged 24 hr at 2°, whereas phenobarbitone inhibits approximately 20 per cent at 100 μM but only with fresh microsomes. This suggests that phenobarbitone inhibits the above microsomal sequence through participation in detoxication reactions whereas Phenergan appears to have some more direct effect on the lipid peroxidation sequence itself. Although such effects based on the transient inhibition of phenobarbitone of enzymic reactions involved in the activation of CCl_4 are possibly the major cause of the results observed here, other and more indirect interactions (e.g. temporary hypothermia) are not, of course, ruled out.

To summarise the findings of this study it can be said that agents that protect the liver against the development of centrilobular necrosis (measured 24 hr after dosing with CCl_4) also prevent the drop in the sum of NADP + $NADPH_2$ (measured 1 hr after poisoning), a finding consistent with the original speculation that stimulated this investigation. However, several agents that protect against the short-term effect are not effective against the long-term reaction. The explanation of this behaviour is probably to be found in the relative rates of accumulation and excretion of the various agents by the liver.

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