Lipid peroxidation in regenerating rat liver

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Received 16 September 1986

Rats entrained to a strictly regulated lighting and feeding schedule have been subjected to partial hepatectomy or a sham operation. In the partially hepatectomised animals the period of liver regeneration is characterised by regular bursts of thymidine kinase activity. Liver microsomes from rats, at times corresponding to maximum thymidine kinase activity, have much reduced rates of lipid peroxidation compared to control preparations: this is due in part to increased levels of lipid-soluble antioxidant at times of maximal DNA synthesis. This temporal relationship between thymidine kinase and lipid peroxidation is consistent with the view that lipid peroxidation is decreased prior to cell division.

Lipid peroxidation *Liver regeneration* *Cell division* *α-Tocopherol* *Thymidine kinase*

1. INTRODUCTION

Lipid peroxidation is considerably less pronounced in many liver tumours compared with normal liver [1–6], and a major contributory factor is an increased concentration of lipid-soluble chain-breaking antioxidant material [6,7]. However, the decreased rate of lipid peroxidation in the liver tumours may be a reflection of an increased proportion of dividing cells in the tumour sample rather than a specific feature of malignancy. For this reason it is of interest to study lipid peroxidation and antioxidant content in regenerating liver after partial hepatectomy, where periodic bursts of DNA synthesis are known to occur [8]. Here, we show that dividing normal liver cells in regenerating liver have a much reduced rate of lipid peroxidation, and an increased content of lipid-soluble chain-breaking antioxidant, analogous to the situation previously observed in liver tumours [6].

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Dedication: This paper is dedicated to Professor Prakash Datta to mark his retirement from the position of Managing Editor, FEBS Letters

2. MATERIALS AND METHODS

2.1. *Chemicals*

[*methyl*-^{3}H]Thymidine was purchased from Amersham International, Amersham, England. All other chemicals were of the highest quality available and were obtained from BDH (Poole, Dorset), Sigma (Poole, Dorset) or Boehringer (Lewes, Sussex).

2.2. *Entrainment of animals and partial hepatectomy*

Male Wistar rats were obtained from Charles River Ltd (Margate, Kent) and were entrained to an inverted lighting and feeding schedule for a period of 3 weeks before partial hepatectomy. The lighting and feeding regimen was that of Hopkins et al. [8,9]: the rats were housed in a windowless room lit from 21.00 h to 09.00 h and they were allowed access to a standard laboratory diet (Expanded Breeder Diet no.3, Special Diet Services, Witham, Kent) from 09.00 h to 17.00 h, cor-

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00145793/86/$3.50

responding to the first 8 h of the dark period. At the time of operation the mean body weight was 270 g. The rats were subject to 65% partial hepatectomy [10] under ether anaesthesia. Sham-operated animals were subjected to laparotomy.

The rats were killed by cervical dislocation at various intervals after operation and the liver quickly removed. Small necrotic stubs were removed from the partially hepatectomised rats before homogenising the liver. The minor lobes of the livers were homogenised and microsomes prepared as described in [11]. Samples of homogenate were taken for analysis of liver triglycerides and polyunsaturated fatty acids (described below). Microsomal pellets were rinsed with 0.15 M KCl and stored at −20°C for up to 60 h before resuspension. Post-microsomal supernatant fractions were taken for the assay of thymidine kinase (EC 2.7.1.21) activity; after removal of floating fat, these fractions were stored at −20°C.

2.3. *Assays of microsomal enzyme activities*

Liver microsomes were resuspended in 0.15 M KCl such that 1 ml was equivalent to 1 g wet wt liver. Cytochrome P-450 and NADPH:cytochrome *c* reductase (EC 1.6.2.4) were measured as in [12]. NADPH/ADP-iron-dependent lipid peroxidation was determined by oxygen uptake [13]. The time (in min) between addition of ADP-iron to the microsomal NADPH suspension and the start of the maximal rate of oxygen uptake was derived from the recorded traces, and is termed the 'induction period' or lag time.

2.4. *Other procedures*

The content of α-tocopherol in microsomal suspensions was measured by an HPLC method [14] after extraction of the microsomes using a method based on that of Burton et al. [14]. The α-tocopherol extraction was as follows: microsomal suspension (1 ml) was mixed with 2 ml SDS (25 mM), 3 ml absolute ethanol and 1 ml *n*-heptane. After rotamixing for 1–2 min, phase separation was achieved by a brief centrifugation and the *n*-heptane phase was used for HPLC analysis. Total microsomal lipid-soluble antioxidant activity was also measured in this extract by the inhibited styrene-oxidation method of Burton et al. [14,15].

Microsomal fatty acids were measured after chloroform-methanol extraction [16]. Thymidine kinase activity was measured in the post-microsomal supernatant fractions essentially by the method of Ives et al. [17] with slight modification: non-phosphorylated [^{3}H]thymidine was removed from the ion-exchange filter paper discs (Whatman DE81) by mounting them in a Millipore filtration apparatus and washing eaach of them with 45 ml of 1 mM ammonium formate and 30 ml distilled water drawn through by suction. Protein was determined in the various liver fractions by the method of Lowry et al. [18].

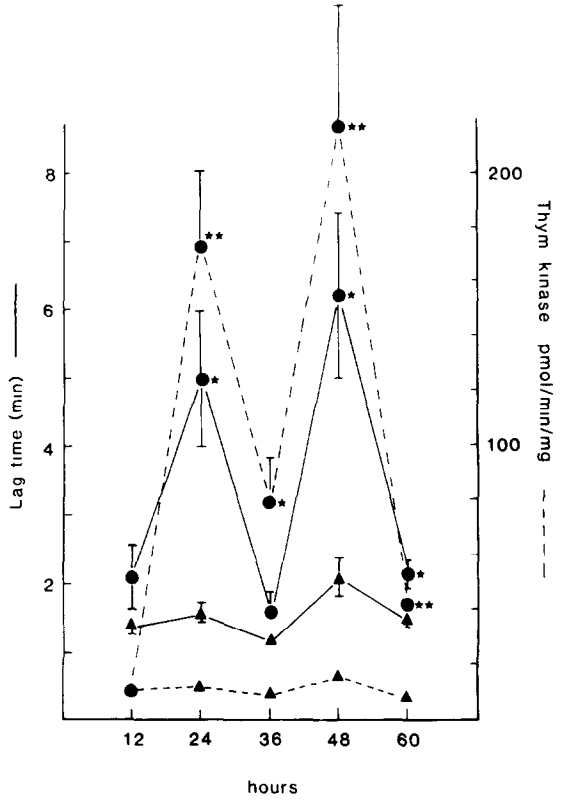

Fig.1. Changes in thymidine kinase and in the lag time (induction period) of lipid peroxidation in liver samples obtained from sham-operated (▲) and partially hepatectomised (●) rats. Mean values are given ± SE. The numbers of rats used for thymidine kinase assays were 3 at 12 h, 9 at 48 h and 6 for other time points; for lag times the numbers of rats were 17 at 24 h, 9 at 48 h and 6 for other points. * $P < 0.01$; ** $P < 0.001$ (Student's *t*-test) for differences between hepatectomised and sham-operated groups.

3. RESULTS

Fig.1 gives results obtained for the activity of thymidine kinase during regeneration, and corresponding data for sham-operated rats. Marked periodicities are observable at 24 h intervals. The lag times (induction periods, see section 2) for NADPH-ADP-iron-stimulated lipid peroxidation in microsomal suspensions included in fig.1 show a similar periodicity to that found for thymidine kinase.

Table 1 gives information on microsomal arachidonic and docosahexaenoic acids, and α-tocopherol in samples prepared from rats that had either had operations for partial hepatectomy or sham operations. There were no marked changes observable in $C_{20:4}$ or $C_{22:6}$ fatty acids in the microsomal fractions isolated from the sham-operated or partially hepatectomised rats. Although there were statistically significant differences in the amount of $C_{20:4}$ between sham and hepatectomised groups at the 36 and 60 h time points, these differences are relatively small and unlikely to affect the rate of lipid peroxidation (for discussion of this see [6]). Indeed, these small decreases in $C_{20:4}$ are at time points when the induction periods of lipid peroxidation are at their minima. The total microsomal fatty acid content did not vary significantly in these experiments (not shown).

The concentration of α-tocopherol in liver microsomes from partially hepatectomised rats shows some periodicity in phase with the changes in lipid peroxidation induction periods, but only at one time point (24 h) is the increased concentration of α-tocopherol in the hepatectomised groups of statistical significance (table 1). Nevertheless, the α-tocopherol content of each microsomal pellet correlated with the induction period in minutes for the appropriate sample ($r = 0.81$; $P < 0.001$). A separate analysis of the total microsomal lipid-soluble chain-breaking antioxidant prepared from normal and regenerating liver demonstrated that α-tocopherol is the major component (the mean ratio of α-tocopherol:total lipid-soluble antioxidant ± SE was 0.99 ± 0.02 for 50 separate samples). Table 2 shows that the activity of NADPH:cytochrome *c* reductase which catalyses the NADPH/ADP-iron-dependent microsomal lipid peroxidation is not significantly different in the two groups at any time point and shows no periodic cycling. Cytochrome P-450 concentrations (nmol/mg protein) are significantly depressed at 48 and 60 h after operation in regenerating liver compared with the sham-operated controls. There were no significant differences observed in microsomal proteins/g wt liver or in total microsomal lipid between regenerating liver samples and sham-operated controls (not shown).

Table 1

Changes in arachidonic acid ($C_{20:4}$), docosahexaenoic acid ($C_{22:6}$) and α-tocopherol in microsomal suspensions prepared from rats that had undergone either a sham operation or partial hepatectomy (HEP)

Time (h)	$C_{20:4}$		$C_{22:6}$		α-Tocopherol	
	SHAM	HEP	SHAM	HEP	SHAM	HEP
24	20.7 ± 0.9 (6)	18.4 ± 0.7 (6)	6.4 ± 0.4 (6)	5.9 ± 0.4 (6)	1.43 ± 0.16 (7)	2.44 ± 0.32 (7)[b]
36	24.4 ± 1.0 (6)	20.0 ± 0.7[c] (6)	6.6 ± 0.4 (6)	6.6 ± 0.5 (6)	1.47 ± 0.07 (7)	1.36 ± 0.08 (7)[e]
48	19.1 ± 1.5 (8)	16.4 ± 2.0 (9)	6.6 ± 0.4 (8)	7.3 ± 0.4 (9)	1.93 ± 0.13 (6)	2.23 ± 0.13 (7)[f]
60	22.9 ± 0.2 (5)	20.4 ± 0.3[d] (6)	8.4 ± 0.1 (5)	9.7 ± 0.4[a] (6)	1.05 ± 0.22 (4)	1.30 ± 0.14 (4)[g]

Mean values are given ± SE; the number of rats used is in parentheses. The values for the fatty acids are given as the percentage contributions of $C_{20:4}$ and $C_{22:6}$ to total microsomal fatty acid content which was 285.8 ± 15.8 μg/mg protein in the sham-operated controls. α-Tocopherol values are nmol per mg microsomal lipid. The *P* values given were calculated using Student's *t*-test: [a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.01$; [d] $P < 0.001$ for sham-operated vs partially hepatectomised groups. Within the hepatectomised group: [e] $P < 0.01$ (36 h vs 24 h); [f] $P < 0.001$ (36 h vs 48 h); [g] $P < 0.002$ (48 h vs 60 h)

Table 2

NADPH:cytochrome *c* reductase activity and cytochrome P-450 content in microsomal suspensions prepared from rats that had undergone either a sham operation or partial hepatectomy (HEP)

Time (h)	NADPH:cytochrome *c* reductase		Cytochrome P-450	
	SHAM	HEP	SHAM	HEP
24	91.4 ± 6.2 (12)	91.1 ± 6.0 (14)	0.50 ± 0.03 (12)	0.48 ± 0.02 (14)
36	85.3 ± 6.1 (6)	86.1 ± 4.5 (6)	0.47 ± 0.02 (6)	0.41 ± 0.02 (6)
48	135.4 ± 20.7 (9)	118.1 ± 15.3 (9)	0.72 ± 0.07 (9)	0.49 ± 0.04[a] (9)
60	101.3 ± 10.4 (6)	92.4 ± 9.2 (6)	0.62 ± 0.03 (6)	0.41 ± 0.01[b] (6)

Mean values are given ± SE; the number of rats used is in parentheses. NADPH:cytochrome *c* reductase activity is given as nmol/min per mg protein; cytochrome P-450 content is given as nmol/mg protein. *P* values were calculated using Student's *t*-test: [a] $P < 0.05$; [b] $P < 0.001$ for sham-operated vs partially hepatectomised groups

4. DISCUSSION

The results for thymidine kinase are very similar with respect to the periodicities first described by Hopkins et al. [8] for thymidine kinase and thymidine incorporation into DNA with the entrained rat model. In the absence of this rigorous entrainment schedule there is a much less marked periodicity in thymidine kinase activity and DNA synthesis, and studies concerned with phenomena that are possibly linked temporally with DNA synthesis become much more difficult to evaluate.

A marked benefit of that schedule of 12 h dark (including 8 h access to food) and 12 h light to studies of the biochemical events in liver regeneration is the pronounced repeated 24 h cycle of DNA synthesis and thymidine kinase activity (see [8] and fig.1). Our data show, for the first time to our knowledge, that corresponding periodicities occur in the induction times of a lipid peroxidation system in liver microsomal suspensions.

Microsomal components that can affect the rate of lipid peroxidation include the content of polyunsaturated fatty acid substrate (in particular $C_{20:4}$ and $C_{22:6}$ fatty acids), the activities of NADPH-cytochrome P-450 reductase, cytochrome P-450 and the presence of antioxidant substances. As already noted in section 3, there were no significant cyclical changes in the NADPH-cytochrome P-450 reductase (measured as NADPH-cytochrome *c* reductase) or cytochrome P-450. The first enzyme mentioned is known to be essential in supplying electrons for the NADPH-ADP-iron-stimulated lipid peroxidation [20] studied here; cytochrome P-450 can act to initiate further radical chains by converting lipid hydroperoxides to peroxyl and alkoxyl radicals [19]. Microsomal fatty acids do not show cyclical variations during liver regeneration when considered as total fatty acid, as $C_{20:4}$ with $C_{22:6}$, or as total bisallylic methylene groups (not shown). In fact, the only component studied that has periodicity of behaviour, and which can be expected to affect lipid peroxidation in the microsomal membrane suspensions, is lipid-soluble chain-breaking antioxidant of which the main component is α-tocopherol. Moreover, since the main periodicity indicated in fig.1 for lipid peroxidation is that of the induction period rather than rate of reaction and since the activity of the enzyme catalysing the initiation of lipid peroxidation does not show periodicity, the implication [15] is that there is a change in antioxidant with little or no change in substrate.

In fact, as mentioned in section 3, there is a correlation between the microsomal concentration of α-tocopherol and the length of induction period of lipid peroxidation. However, the changes in α-tocopherol concentration do not show the same marked periodicity and, in our view, are not sufficient in themselves to account for the modulations in induction period. Rather, α-tocopherol may be

acting together with some other as yet unidentified factor(s) to decrease lipid peroxidation.

Certainly (see section 3), α-tocopherol is the major lipophilic chain-breaking antioxidant in liver microsomes prepared from control and partially hepatectomised rats and this finding is similar to the results obtained for intact rat liver, isolated normal hepatocytes and Novikoff liver tumour [6] as well as for human plasma and erythrocyte membranes [14]. It is known, however, that the antioxidant activity of α-tocopherol is closely inter-related to a number of other hydrophilic antioxidants, both preventive and chain-breaking [15,21–24], for example ascorbate, glutathione, glutathione peroxidase; the changes in these and other antioxidants in regenerating liver are under study.

A number of previous studies have reported a decrease in lipid peroxidation during liver regeneration. In those studies the lipid peroxidation was measured using a variety of procedures, usually at only a few (or one) time points, in homogenates or microsomal suspensions, and without entraining the rats to the schedule used here (e.g. see [2,4,5,25,26]).

No previous report to our knowledge has described the cyclical changes in microsomal lipid peroxidation reported here and so closely associated in time with the periodic bursts of thymidine kinase activity and (see [8]) DNA synthesis. These events may be unrelated in a direct sense, of course: partial hepatectomy is a severe biological insult to the animal and changes consequent upon anaesthesia, operation trauma and decreased blood volume have to be separated from those relating to regeneration. Nonetheless, the close time relationships observed in the periodicities of thymidine kinase activity and peroxidative induction periods are suggestive of inter-linked events, and are consistent with the hypothesis (see [27]) that a decreased lipid peroxidation has some biologically significant role in events leading through DNA synthesis to cell division.

ACKNOWLEDGEMENTS

We are grateful to the Association for International Cancer Research, to the National Foundation for Cancer Research, and the Cancer Research Campaign for financial assistance for this project.

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