Circadiane Rhythm of Cell Proliferation in Rat Liver: Synchronization by Feeding Habits

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A diurnal rhythm of cell proliferation exists in the liver of young rodents and in the growing liver of rats treated with α-hexachlorocyclohexane (α-HCH). The timing of the rhythm is dependent on the time of feeding but independent from the lighting regimen. This suggests that under natural circumstances the light-dark rhythm synchronizes the rhythm of hepatocyte proliferation by controlling the animals' feeding habits.

A diurnal rhythm in the rate of liver cell proliferation has been observed in many studies on laboratory animals. In the liver of young rodents and in regenerating rodent liver, mitotic activity was found to be high during the day, and low during the night [1–6]. Circadiane fluctuations exist also in the rate of hepatic DNA synthesis [2, 4, 6, 7]. Halberg *et al.* have shown that on inversion of the lighting regimen the rhythms of DNA synthesis and mitosis shift in phase by 12 hours and concluded that environmental lighting is the dominant synchronizer [6]. However, up to now the mechanism by which rhythmic light-dark changes effect the synchronization of liver cell proliferation is not known.

In the present investigation, hepatocyte proliferation was studied in normal young rats and in rats, whose liver had been stimulated to grow by prior administration of α-hexachlorocyclohexane (α-HCH). This chemical agent, an isomer of the insecticide lindane (γ-HCH), leads to a pronounced hypertrophic and hyperplastic response in the liver without producing any detectable damage [8–11]. The results reported below indicate that the stimulatory effect of α-HCH greatly varies with the time of day. This makes the drug a valuable tool in the search for factors which control the circadiane rhythm of cell proliferation.

Materials and Methods

Female SPF-rats of Wistar strain obtained from Zentralinstitut für Versuchstierzucht, Hannover, Germany, were adapted to controlled lighting and feeding schedules as specified in the legends. The food used was a commercial diet (Altromin R 10); water was always available in all experiments. α-HCH (a gift of Merck, Darmstadt) was dissolved in olive oil (2% or 3%) and administered intraperitoneally or orally by stomach tube at different times of the day.

[^{3}H]thymidine (6.5 Ci/mmol, NEN Frankfurt) was used to measure DNA synthesis. It was injected into a tail vein at constant intervals after administration of α-HCH. The rats were killed by decapitation, and the liver was quickly excised, blotted, weighed and chilled after a specimen had been taken for histological analysis. For determination of DNA the liver was homogenized in 9 volumes of cold 0.2 N perchlorid acid. The sedimentable material was washed with the same acid until the supernatants contained no radioactivity; DNA was then dissolved by heating for 30 min at 75 °C in 0.5 N perchloric acid and was assayed by the Burton procedure [12]. Radioactivity was measured in a Tricarb liquid scintillation spectrometer (Beckman). From each liver two assays were run in parallel.

For histological autoradiography, specimens of liver were fixed in formalin and embedded in paraffin. Sections, 5 μm thick, were covered with stripping film (Kodak AR 10) or liquid emulsion (Ilford G 5). Exposure time was 5 weeks. The percentage of labelled cells and of mitoses was determined by counting at least 2,000 cells of each liver.

Results and Discussion

Fig. 1 shows the diurnal variations in the proliferative response of the liver to α-HCH. The rate of thymidine incorporation into DNA (dpm/μg DNA, Fig. 1 a) shows a peak at 10.00 a.m. and has a trough at 22.00 hours. The number of labelled cells (= cells involved in DNA synthesis)

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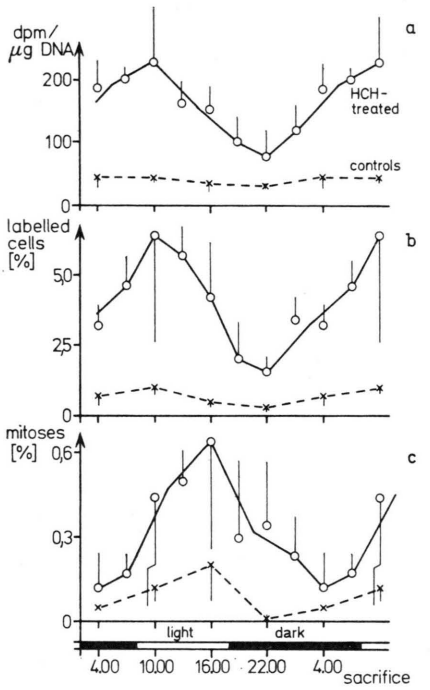

Fig. 1. Diurnal variations of liver cell proliferation. Rats were adapted for 2 weeks to a controlled light-dark rhythm with light periods from 8.00 to 18.00 hours as indicated on the abscissa. Food was given ad libitum. The rats were used when they weighed about 130 g. ×——×: Untreated control rats. ○——○: 250 mg/kg α-HCH were administered orally in 2 doses 50 and 26 hours before the animals were killed. All rats received 0.5 mCi/kg [^{3}H]thymidine 2 hours prior to sacrifice. Abscissa indicates day time of sacrifice. Each point is the mean of 4—8 animals. Vertical bars indicate standard deviation. In Figs 1 a and b data from treated rats differed significantly ($p<0.05$ or less) from controls at all time points. Data obtained from HCH-treated rats at 10.00 and 22.00 hours were also significantly different ($p<0.02$). Mitotic activity (Fig. 1 c) at 13.00 and at 4.00 hours differed significantly ($p<0.01$) in the treated animals (t-test, Student).

fluctuates in an almost identical temporal pattern (Fig. 1 b). In contrast, maximum and minimum of mitotic activity are shifted in phase by about 6 hours (Fig. 1 c); the shift was expected since DNA synthesis precedes cell division in the replicative cycle. These results consistently demonstrate the existence of a diurnal rhythm in the ability of the liver to respond to α-HCH by cell proliferation.

Comparing the results obtained from HCH-treated rats with those from untreated controls (Fig. 1) one can see that a. α-HCH markedly elevates the proliferative activity which is in accordance with earlier reports from this laboratory [8, 9], that b. the diurnal rhythm appears to be pronounced in the treated animals, and that c. minima and maxima coincide in time in both treated and untreated rats. Similar temporal patterns as in the present work were observed in earlier studies on intact or regenerating rodent liver [1–7]. This indicates that the appearance and the time-course of the rhythm of cell proliferation do not depend on the nature of the growth stimulus used, but rather are controlled by environmental factors such as the light-dark rhythm.

Some observations suggest that food intake, too, might have some importance in the timing of the diurnal rhythm [7, 13]. Since food consumption shows circadiane periodicity [14–16], we decided to test, to what extent cyclic variations of food intake might

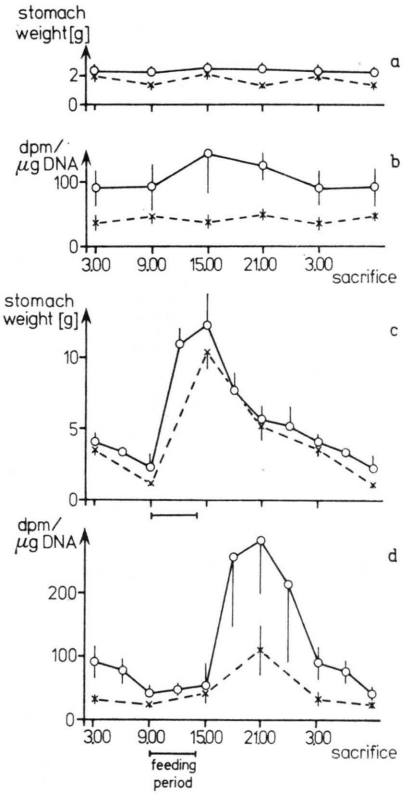

Fig. 2. Stomach weights and hepatic DNA synthesis after continuous lighting. Rats were adapted to continuous lighting for 4 weeks. The animals were used when they weighed about 100 g. ×——× Untreated control rats. ○——○: 150 mg/kg α-HCH were injected i. p. 31 hours before killing. All rats received 0.5 mCi/kg [^{3}H]thymidine 1 hour prior to sacrifice. Stomach weights (with content) are given in percent of body weight. Each point is the mean of 4—5 animals, vertical bars indicate standard deviation.
a, b: Food was given ad libitum.
c, d: Food was given from 9.00 to 14.00 hours (horizontal bars). Differences between data from treated rats are not significant in Fg. 2 b ($p>0.05$); in Fig. 2 d the increase between 15.00 and 18.00 or 21.00 hours, resp., is significant in control and treated rats ($p<0.01$).

contribute to the control of the timing of hepatic DNA synthesis.

For this purpose we excluded the influence of the light-dark rhythm by adapting rats to continuous lighting for 4 weeks. One group of animals received food ad libitum throughout this period. After sacrifice, stomach weights (with contents) were recorded to indicate feeding habits. Fig. 2 a shows that stomach weight, on the mean, remains constant throughout the day, and no significant diurnal variations in the rate of thymidine uptake by DNA are present in control or HCH-treated series of animals (Fig. 2 b). This indicates that continuous lighting abolishes the synchronous fluctuations of hepatic DNA synthesis and of food intake.

Another group of rats under continuous light had access to food for only five hours per day. Fig. 2 c shows that the stomach weights increase sharply after begin of feeding. When food is withdrawn, stomach emptying proceeds quickly until about 21.00 hours and slows down thereafter. Liver weights raise to a maximum at 21.00 hours and then decline (not shown). The rate of DNA synthesis as indicated by [^{3}H]thymidine incorporation (Fig. 2 d) remains on a low level until 15.00 hours. Then, in the treated rats a steep increase occurs which lasts for about 7 hours, *i.e.* the duration of the DNA synthetic period, and then declines. Initiation of DNA synthesis appears therefore to be restricted to a few hours. In control animals, [^{3}H]thymidine uptake by hepatic DNA follows a similar time pattern as in HCH-treated rats (Fig. 2 d, dashed line).

It is interesting to note that the peak of DNA synthesis (Fig. 2 d) coincides in time with the end of the rapid phase of stomach emptying (Fig. 2 c) and with the maximum of liver weight. This supports the idea that processes connected with food intake and digestion facilitate the DNA synthetic response to α-HCH. Initiation of DNA synthesis occurs between 15.00 and 18.00 hours, *i.e.* 6 – 9 hours after onset of feeding (Fig. 2 d). In contrast, α-HCH induces DNA synthesis only after a lag period of at least 16 hours [8]. This suggests that the facilitating effect of feeding is active several hours after triggering the preparation for DNA synthesis with α-HCH.

The present results also show that rhythmic variations of food intake may act as the only synchronizer of hepatocyte proliferation, if the influence of lighting changes is excluded. In a final experiment we checked whether, under natural conditions, it is the light-dark rhythm or the rhythm of food consumption, which acts as the real Zeitgeber of hepatocyte proliferation. To answer this question rats were adapted to a normal day-night rhythm, but received food only during the day. If the light-dark change was the dominant synchronizer, then maximal DNA synthesis would be expected in the morning; if the feeding-fasting rhythm was the main synchronizer, peak DNA formation should occur in the evening.

Table. Stomach weight, liver weight, and hepatic DNA synthesis in rats fed during the light period. Rats were adapted for 3 weeks to a controlled light–dark rhythm with lights from 9.00 to 21.00 hours; food was available from 9.00 to 14.00 hours. The rats killed at 10.00 hours were not fed on the day of sacrifice. The animals were used when they weighed about 100 g. α-HCH-treatment was as described in the legend to Fig. 2. 0.1 mCi/kg [^{3}H]thymidine was injected 1 hour before sacrifice. Stomach and liver weights are expressed in percent of body weight. Standard deviations are given. The differences in the specific activities of DNA from control and treated rats and from rats killed at 10.00 and at 22.00 hours all are significant ($p<0.05$ or less).

Rats	α-HCH	Sacrifice	Stomach Weight	Liver Weight	[dpm/ μg DNA]
4	–	10.00	1.24 ± 0.03	3.6 ± 0.25	3.8 ± 1.3
3	–	15.00	5.84 ± 0.37	4.5 ± 0.13	4.4 ± 0.66
4	–	22.00	3.36 ± 0.88	5.0 ± 0.25	11.6 ± 5.1
6	+	10.00	1.51 ± 0.15	4.0 ± 0.19	8.4 ± 2.5
6	+	22.00	5.92 ± 1.28	5.2 ± 0.24	33 ± 8.8

The result of the experiment as summarized in the table supports the latter alternative by showing that the rate of [^{3}H]thymidine incorporation into DNA was considerably higher in the evening than in the morning, both in control and HCH-treated animals. A consistent observation was recently reported from another laboratory [13].

Our results indicate that diurnal variations of food consumption mainly control the timing of the diurnal rhythm of hepatocyte proliferation in untreated and HCH-treated rats. Environmental lighting which was shown earlier [6] to be the dominant synchronizer appears to act merely by controlling the feeding habits of the animals. Moreover, the possibility to induce an almost synchronous wave of DNA synthesis in the liver is of obvious practical interest for investigations of the replicative cycle.

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