ISOPROTERENOL-INDUCED PHASE SHIFTS IN CIRCADIAN RHYTHM OF MITOSIS IN MURINE CORNEAL EPITHELIUM

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INTRODUCTION

The stimulation of DNA synthesis and subsequent mitosis by isoproterenol (IPR) was thought to occur only in the parotid gland and kidney (1, 2). In the parotid gland, the peak in DNA synthesis occurred 28 h after a single injection of IPR. It recently was demonstrated in mice that the duodenum also responds to a single intraperitoneal injection of IPR (3). The total uptake of [^{3}H]thymidine ([^{3}H]Tdr) into all three of the above-mentioned organs fluctuated with a circadian frequency in both saline- and IPR-treated animals. In the duodenum the amount of [^{3}H]Tdr uptake in the IPR-treated animals was dependent on the phase of the mouse circadian system at which the IPR was injected: during one phase it increased, at another it decreased and at still another phase, no statistically significant effect on total [^{3}H]Tdr uptake was noted.

Recently it was reported that a single injection of IPR resulted in an "inhibition" of mitosis 24 h after injection and a "stimulation" at 48 h in rat lens epithelium (4, 5). The present report demonstrates that the results obtained from a single injection of IPR can be misleading without time series analysis. IPR changes the phasing and thus alters the profile of the normal circadian rhythm.

MATERIALS AND METHODS

All animals were standardized for 7 days before each study. The standardization included keeping subgroups of five to six mice per cage with five cages in each light-tight, ventilated, isolation chamber. Each chamber was maintained at 23 ± 2°C. The chamber was illuminated with fluorescent light from 0600 to 1800 (CST) and completely darkened from 1800 to 0600 (CST). The maximum light within each cage was approximately 10 foot candles. Rockland mouse chow and water were available ad libitum.

In the first experiment, BFD_1 mice averaging 22 ± 3 g in body weight were used. Initially 72 mice were injected intraperitoneally in subroups of 6, either with 7.5 mg of freshly dissolved isoproterenol (IPR) in 0.75 ml of distilled water or with 0.75 ml of saline at 0900, 1300, 1700, 2100, 0100, and 0500. Exactly 28 h after injection (see reference 3), each mouse within a subgroup was killed by cervical dislocation and immediately fixed in 10% buffered formalin.

In the second experiment, Swiss Webster mice averaging 24 ± 3 g were used. Initially, 90 standardized mice were injected intraperitoneally with 7.5 mg of IPR at 0900 and then divided into 18 subgroups of 5 mice each. The first subgroup was killed 12 h later at 2100, and thereafter a subgroup was killed every 4 h throughout a 68 h span. Identical subgroups of saline-injected mice served as controls. Another 85 standardized mice were injected with 7.5 mg of IPR at 2100, and thereafter a subgroup of 5 mice was killed every 4 h for 64 consecutive h beginning 4 h (0100) after the injection (2100).

Corneas were removed, stained with hematoxylin, and prepared as whole mounts. In each specimen the number of mitotic figures was counted in at least 5,000 cells. The mean mitotic index of each sampling period was expressed as the number of mitoses per 1,000 cells.

RESULTS

The data obtained from the first experiment are illustrated in Fig. 1. The solid line demonstrates a prominent circadian rhythm in mitosis in the corneal epithelium of the control mice. The peak mitotic index occurred between 0500 and 0900 with the highest recorded mean mitotic index of 10.8 ± 1.0 at 0900; the lowest mean of 2.1 ± 0.5 was recorded at 2100. The overall 24 h mean was 6.1 ± 0.8. The peak mean value is significantly different from the low mean value ($P < 0.001$). The range of change between the lowest and highest recorded means was approximately 400%. This confirms previous reports of a circadian rhythm in the mitotic index of corneal epithelium in rodents (6–8).

The dashed line in Fig. 1 represents the data obtained from mice injected with IPR every 4 h during one 24 h period, and killed exactly 28 h later (kill times, not injection times, are plotted). Mice injected with IPR at 0100, 0500, and 0900 demonstrate a statistically significant depression in the mitotic index 28 h later at 0500 ($P < 0.001$), 0900 ($P < 0.001$), and 1300 ($P < 0.01$). The injections of IPR made at 1300, 1700, and 2100 had no statistically significant effect on the mitotic index seen 28 h later at 1700, 2100, and 0100. The overall 24 h mean for the IPR-treated group was 3.0 ± 0.7. A similar observation has been recorded in a study on the effect of IPR on duodenal mitosis (9).

The data obtained from the second experiment are illustrated in Fig. 2. The solid line

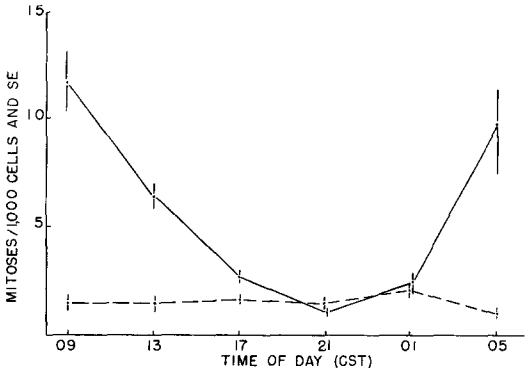

FIGURE 1 Mean mitotic index (number of mitoses per 1,000 cells and the standard error of the mean) in the corneal epithelium of saline-injected (solid line) and isoproterenol-injected (dashed line) standardized mice. Each point represents six mice. The points plotted are kill times, not injection times. The mice were injected intraperitoneally with saline or IPR at 0900, 1300, 1700, 2100, 0100, and 0500. The mice were killed exactly 28 h after injection. For example, mice receiving IPR at 1300 were killed at 1700 on the next day.

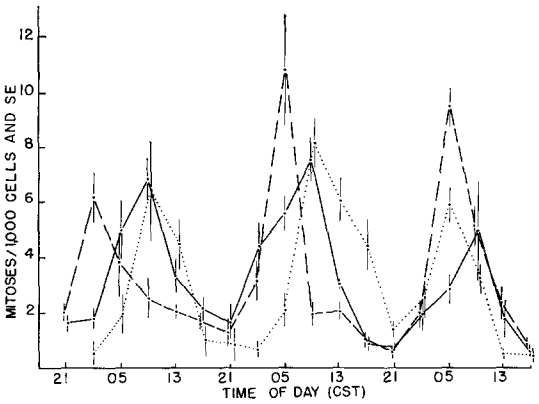

FIGURE 2 Mean mitotic index (number of mitoses per 1,000 cells and the standard error of the mean) in the corneal epithelium of standardized mice injected with saline (solid line), with isoproterenol at 0900 and killed in subgroups of five mice each every 4 h beginning 12 h (2100) after IPR injection (dashed line), and mice injected with IPR at 2100 and killed in subgroups of five mice each every 4 h beginning 4 h (0100) after the IPR injection (dotted line). The points plotted are kill times.

represents the natural rhythmic pattern in the mitotic index in corneal epithelium of control animals over a 68 h span. The phasing is identical to that seen in Fig. 1; the peak mitotic index consistently occurred at 0900 and the trough at 2100. The dashed line in Fig. 2 represents the data obtained from mice injected with IPR at 0900. The mitotic index subsequently was monitored at 4-h intervals for a 68 h time span beginning at 2100 (12 h after injection). The data revealed an advance in phasing of the peak of the rhythm by at least 8 h during the 1st day and by 4 h on the 2nd and 3rd days of the experiment. The trough did not shift but did become more extended in time. Because of the shift in phasing, there is a statistically significant difference between control and IPR-injected animals at 0100 ($P < 0.01$) and 0900 ($P < 0.05$) on the 1st day. The same applies at 0500 ($P < 0.05$) and 0900 ($P < 0.05$) during the 2nd day. On the 3rd day, only one time point, 0500, demonstrated a statistically significant difference ($P < 0.001$) in mitotic indices between IPR-treated and control animals.

The dotted line in Fig. 2 illustrates the data obtained from the mice injected with IPR at 2100 and killed every 4 h thereafter for 64 consecutive h. The peaks occurred at the same time point as the controls for the first 2 days, but on the 3rd day the peak was advanced by 4 h. On day 1, there was a statistically significant depression in the mitotic index at 0100 ($P < 0.05$). On day 2 there was a significant depression at 0100 ($P < 0.05$) and 0500 ($P < 0.01$), and a significant increase in mitotic index at 1300 and 1700 ($P < 0.05$ in both cases). On the 3rd day, there was a significantly higher mitotic index in the IPR subgroup at 0500 ($P < 0.05$).

The overall mean mitotic index for each group over a 68 h period was 3.2 ± 0.4 for the controls, 3.3 ± 0.6 for IPR at 0900, and 3.1 ± 0.5 for IPR at 2100. There were no statistically significant differences between these means.

DISCUSSION

It could be concluded from Fig. 1 that IPR abolishes the circadian rhythm characteristic of the mitotic index in corneal epithelium. However, when one takes into consideration the basic rhythmic nature of cell division in corneal epithelium and the collective results obtained from the second part of the study, it is quite likely that somewhere in between each of the 28-h spans between injection of IPR and killing, there occurred a peak in the mitotic index. Irrespective of what did happen during each 28 h period, it must be concluded that one cannot reliably accept the conclusion that a single injection of IPR significantly depresses the overall mitotic activity or abolishes the circadian rhythm.

The second phase of the study demonstrates that a single injection of IPR only advances the phasing of the peak (by as much as 8 h on the 1st day and 4 h on days 2 and 3) and that it extends the troughs to periods as long as 16 h, especially if the injection is made at 0900. Apparently, when IPR is injected at the time of peak mitotic index (0900), it accelerates progress through the cell cycle, resulting, on the subsequent day, in a mitotic peak 8 h earlier than normal. The effect of a phase advance still is evident on the 2nd and 3rd days. The delayed effect on the advance in phase of the peak on the 3rd day of the IPR at 2100 group is of interest because it also demonstrates that the phase of the animal's circadian system at which the drug is given may affect the ultimate response. These results are different from those obtained when cytosine arabinoside, an inhibitor of DNA synthesis, was injected at 1800. In this experiment. the peak mitotic index of corneal epithelium was delayed 4 h and occurred at 1300 instead of at 0900 (10).

These studies suggest that infrequent sampling during the 24 h period and failure to consider the circadian time structure of the organism can lead to erroneous conclusions regarding the effect of IPR. For example, if one injected mice at 0900 (dashed line in Fig. 2) on 1 day and compared mitotic indices with saline-injected animals at 0900 on the following 3 days, a statistically significant depression in mitotic index would be obtained during the first 2 days in the IPR-injected animals. On the 3rd day the conclusion would be that the mitotic index had returned to normal. On the other hand, if IPR was injected at 2100 (dotted line in Fig. 2) on 1 day and sampled at 0900 on the following morning and thereafter at the same hour for 2 consecutive days, the conclusion would be that IPR had no effect on mitotic index. If one sampled only at 0500 subsequent to this 2100 injection time, the conclusion would be that IPR had no effect on the 1st day, but significantly depressed the mi-

totic index on the 2nd day and significantly enhanced it on the 3rd day. If one further explores the data in Fig. 2, a number of possible additional pitfalls become apparent. It should become obvious that the scientifically correct picture of what IPR actually does depends on a thorough analysis and awareness of circadian variation. The data also indicate the necessity for considering the time structure of an experimental animal in any investigation involving the effect of different drugs on cell division or when studying the mechanism of control of cell division.

SUMMARY

The effect that a single injection of IPR has on the normal circadian rhythm in mitosis in corneal epithelium was studied. In the first experiment, it appeared that IPR had depressed the mitotic index to trough levels 28 h after injection, irrespective of the phase of the mouse circadian time structure during which IPR was injected. However, in the second experiment, a single injection of IPR at 0900 advanced the phasing of the peak of the normal rhythm by 8 h on the 1st day and by 4 h on both the 2nd and 3rd days. A single injection of IPR at 2100 advanced the phasing of the peak in corneal mitosis by 4 h only on the 3rd day after the injection. In this experiment, the troughs did not demonstrate a shift in phasing as the peaks did.

The phase shifts and profile alterations that result in the natural rhythm after the administration of IPR could lead one to erroneous conclusions. With infrequent or single sampling intervals, it becomes obvious, when one makes comparisons with saline-injected controls, that there are times when IPR seems to inhibit, stimulate, or have no significant effect on corneal mitoses.

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