Peptides, Vol. 8, pp. 347–353. © Pergamon Journals Ltd., 1987. Printed in the U.S.A. 0196-9781/87 $3.00 + .00

Effect of Epidermal Growth Factor (EGF) on [^{3}H]TdR Incorporation Into DNA in Ad Lib Fed and Fasted CD2F1 Mice

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Received 30 June 1986

SCHEVING, L. A., T. H. TSAI, L. E. SCHEVING AND W. S. HOKE. *Effect of epidermal growth factor (EGF) on [^{3}H]TdR incorporation into DNA in ad lib fed and fasted CD2F1 mice.* PEPTIDES 8(2) 347–353, 1987.—The effect of EGF on the incorporation of [^{3}H]TdR into DNA (DNA synthesis) was determined in the esophagus, liver, pancreas, and kidney in mice standardized to 12 hours (hr) of light alternating with 12 hr of darkness. A question asked was whether intraperitoneally administered EGF could alter the circadian patterns of DNA synthesis in these organs. The most marked effects of EGF were: (1) an increase in DNA synthesis but only after a specific duration of time after treatment, ranging from 8 to 23 hr, which differed for each tissue, (2) a similarity in the response of the esophagus in both ad lib fed and fasted mice, but not in the response of the liver, where the stimulatory effect of EGF observed in fed mice was dramatically reduced in fasted ones, and (3) an advance in the phasing of the circadian rhythm in DNA synthesis of the esophagus by about 12 hr. In addition, no sex differences in fasted animals were found under the conditions of this study.

Epidermal growth factor Pancreas Esophagus Liver Kidney Circadian Rhythm Mice Fasting

THE mitogenic effects of epidermal growth factor (EGF) have been studied extensively *in vitro*, but to a lesser extent *in vitro*. Moreover, there are discrepancies in the described organ responses to EGF in the *in vivo* studies done to date [1–2, 7, 11, 16–18, 22]. For example, we originally reported that EGF consistently inhibited DNA synthesis in the pancreas within 12 hours after injection [18]. In contrast, others reported that multiple injections of lower dosages of EGF over a longer span of time stimulated pancreatic DNA synthesis and led to increased total DNA and organ weight [7]. To resolve these discrepancies and further characterize the role of EGF *in vivo*, we have now studied the effect of EGF on DNA synthesis over 38 hours after injection in four diverse organs—the esophagus, pancreas, liver, and kidney. In addition, because fasting reduces DNA synthesis in the esophagus and liver, we also have studied their response to EGF during a 59 hour fast.

METHOD

Study 1

Six-week-old CD2F1 male and female mice were received from Simonsen Laboratories, Gilroy, CA, on March 25, 1984. The animals were subdivided into groups of five per cage, and four or five such cages were placed in sound-attenuated, temperature-regulated isolation chambers. Each chamber was illuminated from 0700–1900 hr daily. Food and water was available ad lib. The animals were not intentionally disturbed, except that dirty cages were replaced with clean ones once each week on the same day. When ten weeks old, the mice were subdivided as follows: (1) ad lib fed male mice and (2) fasted male and female mice. The fed male mice and fasted male or female mice were injected intraperitoneally with either 0.2 ml of saline (controls) or with mouse EGF (10 μg/mouse/injection) at 0800, 1200, and 1600 clock hr. The rationale for treating at these times is described in the Discussion section. The EGF was generously provided by Dr. Harry Gregory of The Imperial Chemical Industries of Great Britain and was determined to be at least 95% pure by SDS-PAGE. Since food was removed from the cages of the fasted animals at 1600 clock hr of the previous day, these animals had been fasted for a total of 16, 20, and 24 hr at the times of saline or EGF injection. These animals had no access to food, feces, or bedding, but were provided with ad lib access to water.

Subgroups of five fed male mice that had either been injected with saline or EGF were killed at 1600, 2100, 0200, 0700, 1200, 1700, and 2200 clock hours, or 8, 13, 18, 23, 28, 33, and 38 hr, respectively after the initial injection. Sub-

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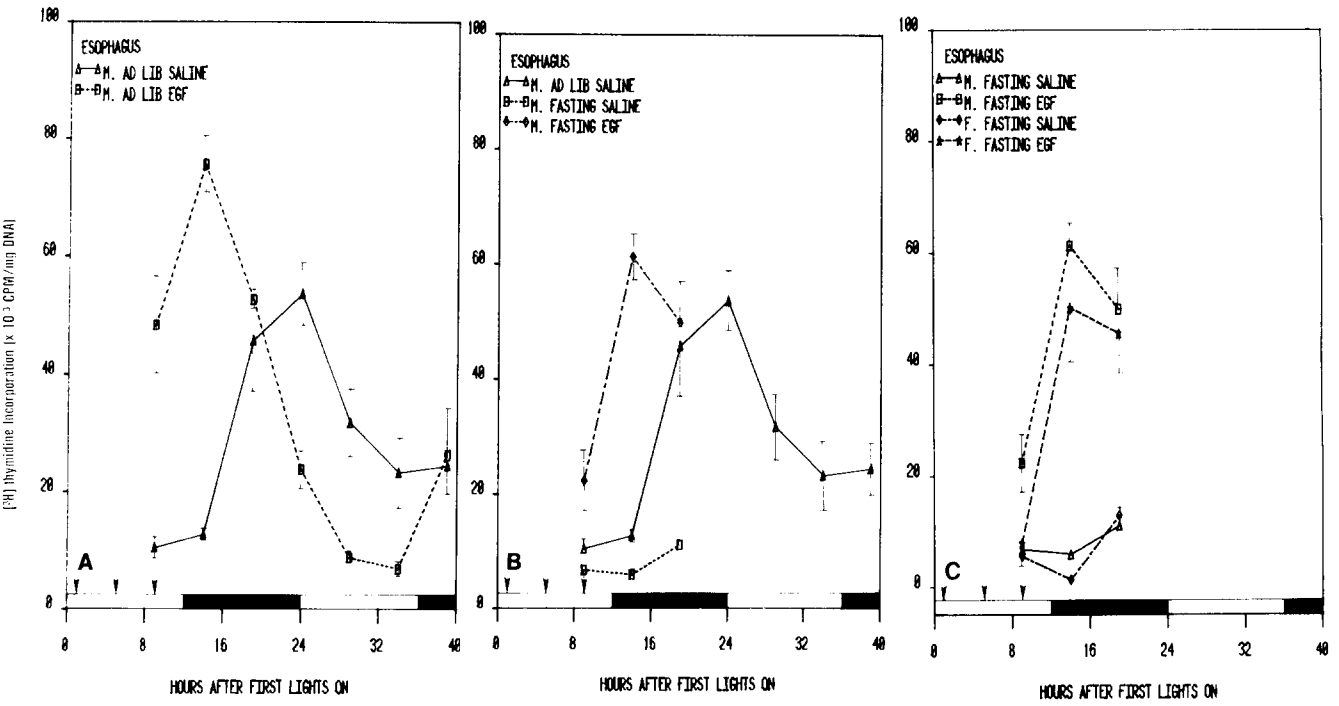

FIG. 1. Panel A illustrates the effect of epidermal growth factor (EGF) or saline on [^{3}H]TdR incorporation into DNA of the esophagus of ad lib fed CD2F1 male mice. Each mouse received either 0.2 ml of saline or 10 μg of EGF at each of the 3 time points or at 1, 5, or 9 hr after the lights came on. Injection times indicated by arrows. Each time point represents a mean and standard error based on five animals. The animals were killed at 8, 13, 18, 23, 28, 33, and 38 hr after the initial treatment with EGF or saline. The horizontal axis marks the light-dark cycle to which the animals were standardized. Lights automatically came on at 0700 and went off at 1900. Panel B depicts, in addition to the ad lib fed data, the response to EGF in three groups of male animals in which the first group had been fasted for 24 hr, the second for 29 and the third for 34 hr. Panel C depicts the same fasting data shown for males in Panel B, but also compares the same response in similarly standardized fasted female littermate mice.

groups of five fasted male mice that had been injected with either EGF or saline were killed at 1600, 2100, or 0200 clock hours, or 8, 13, or 18 hr after the initial injection. At the kill times, these mice had been fasted for total periods of time of 24, 29, or 34 hr. It should be noted that the subgroups of fasted or fed mice killed at 1600 clock hours received only two courses of EGF or saline before killing.

In this first study, there were no ad lib fed females to compare with ad lib fed males; however, there were 3 subgroups of fasted females injected with either saline or EGF, killed at 8, 13, or 18 hr after the initial injection and treated identically to the fasted male mice.

Thirty minutes prior to killing, each mouse was injected intraperitoneally with 25 μCi [^{3}H]-thymidine (25 Ci/mmol) obtained from Amersham, Chicago, IL. Work was performed quickly and quietly. During the dark span, all injections were done under a dim red light (of about 0.5 lux at the level of the mouse eye) and the mice were returned immediately to the dark. After the mice were killed by rapid cervical dislocation, the thoracoabdominal cavity was opened and the carcasses were fixed in 10% buffered formalin solution for two weeks. The incorporation of [^{3}H]-TdR into DNA was compared in several parts of the digestive tract and in the pancreas, liver, spleen, thymus, bone marrow, kidney, lung, and corneal epithelium for each of the treatment groups. The methodology used is described elsewhere [4]. This paper reports the data obtained from the esophagus, pancreas, liver, and kidney.

Data were subjected to conventional statistical tests such as analysis of variance (ANOVA) and the Student *t*-test.

Study 2

A second investigation was carried out exactly as described in study 1 above, except that the fasting span was increased and only female mice were used. Thus, this study included ad lib fed female mice, but no male fed mice.

On 5/8/85, 160 five-week old CD2F1 female mice arrived from Southern Animal Farms, Prattville, AL. They were subdivided into groups of five mice per cage and standardized to 12 hr of light alternating with 12 hr of darkness. On 6/3/85 at 9 hours after the beginning of the lights on (9 HALO) food was removed from the cages of 80 animals. The remaining 80 mice continued on the ad lib diet. Fasted mice did not have access to feces or bedding, wire bottom cages were used.

On the next day, the ad lib fed and fasted female mice were injected with either saline or EGF successively at 1, 5, and 9 HALO (0800, 1200, and 1600 clock hours CST). At each injection time, each mouse received either 0.2 cc of saline or 10 μg EGF in 0.2 cc of saline. Thus, each EGF-treated mouse received a total of 30 μg EGF. At 9 HALO, five EGF and five saline-treated fed mice were killed along with comparable groups of fasted mice. Moreover, 30 min prior to killing each mouse was given [^{3}H]-thymidine as described in study 1. Groups of mice were therefore treated

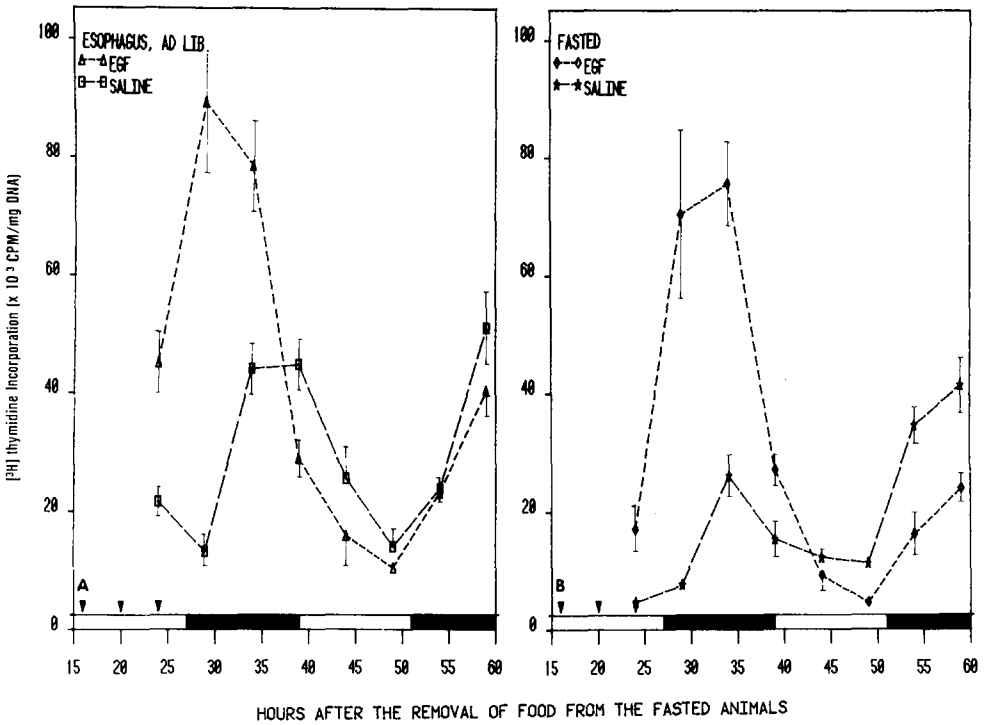

FIG. 2. See legend in Fig. 1 for detailed explanation; note that this study used female mice and a longer sampling time after injection.

and killed in the same manner at five hr intervals or at 29, 34, 39, 44, 49, 54 and 59 hr after the beginning of the fast. Tissues were handled in the same way as in the first study; however, the only tissues studied were the esophagus and liver. The pancreas and kidney were not studied.

In summary, the major differences in the two studies were that animals were obtained from different sources and sampling times differed in frequency and total length.

RESULTS

Esophagus

In the first study, EGF treatment brought about increases of 360% and 491% in DNA synthesis in ad lib fed mice at 8 and 13 hr after the initial injection (Fig. 1A). This confirms an earlier study [16]. The initial increases in DNA synthesis brought about by EGF treatment were followed by significantly lower values in DNA synthesis in the EGF-treated mice at three of the subsequent sampling times. The initial increases were followed by 56%, 72%, and 70% depressions in DNA synthesis at 23, 28, and 33 hr after the initial injection. Thus, the peak of the circadian rhythm in DNA synthesis in the EGF-treated mice was advanced by about 12 h in the ad lib fed mice. Moreover, because the initial increases were followed by actual depressions in incorporation at later sampling times, the overall values for DNA synthesis did not differ significantly between groups.

In the first study, in the fasted male mice, EGF treatment brought about increases ($p<0.001$) of 233% and 939% in DNA synthesis at 8 and 13 hr after the intitial EGF injection (Fig. 1B). Fasting reduced the level of DNA synthesis in this organ, abating or at least delaying the peak of the circadian rhythm of DNA synthesis in the saline-treated mice (Figs. 1B and C) [19]. In the first study, in the fasted female mice, a similiar pattern of response between the sexes was observed (Fig. 1C).

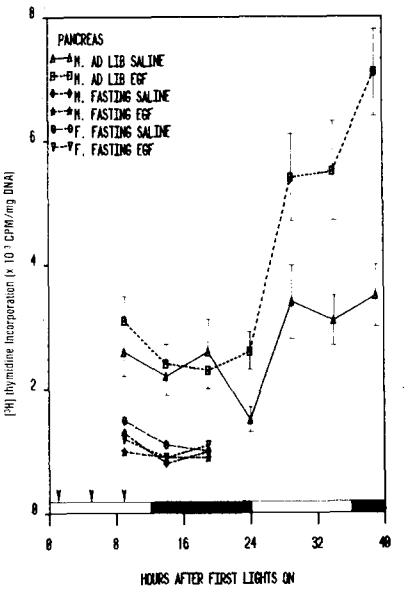

FIG. 3. Data obtained from the pancreas of the same animals. Panel construction as in Fig. 1.

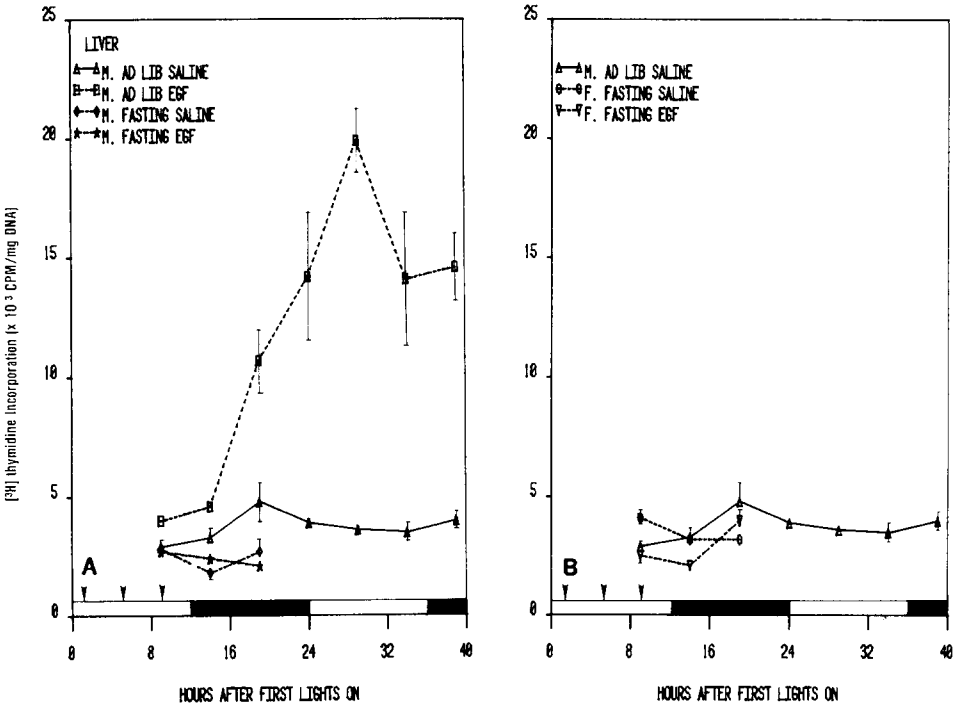

FIG. 4. Data obtained from the liver. Panel construction as in Fig. 1.

In the second study, EGF treatment brought about statistically significant increases of 108%, 572%, and 78% in DNA synthesis in fed female mice at 8, 13, and 18 hr, respectively, after the initial EGF injection. There was also a significant decrease ($p<0.01$) in DNA synthesis of 36% of saline-control values at 23 hr (Fig. 2A). Overall, however, as in study 1, there were no significant differences in time-averaged DNA synthesis rates between the EGF and saline-treated animals. As mentioned previously, this study used ad lib or fasted female mice. Compared to the male ad lib mice in study 1 (Fig. 1A), the esophageal pattern of response in the ad lib female was similar to that in the male.

Liver

In the first study, EGF treatment brought about sustained and significant increases in DNA synthesis in the male ad lib fed mice (Fig. 4A). The increase in DNA synthesis recorded at 8, 13, 18, 23, 28, 33, and 38 hr after the initial injection of EGF were 38%, 39%, 123%, 264%, 453%, 303%, and 265%, respectively; all were statistically significant ($p<0.008$). In contrast to the esophagus, the overall mean levels of DNA synthesis in the EGF-treated mice compared to saline-treated mice showed a 216% increase ($p<0.001$) over the 30 hr sampling span. In the first study, EGF had no statistically significant effect on DNA synthesis in either fasted male or female mice except for statistically significant decreases in DNA synthesis in EGF-treated fasted females at 8 and 13 hr after injection (Fig. 4B). The same was not observed for male animals.

In the second study, in EGF-treated fed animals, the DNA synthesis rates were significantly increased (Fig. 5A). If one compares the hepatic and esophageal response of the male ad lib animals used in the first study with that of the female ad lib animals used in the second study, it appears that the hepatic response of the female mice is more rapid than that of the males. The peak hepatic occurs broadly at 18 and 23 hr after the initial injection in the first case and at 28 hr in the second. This is in contrast to the greater similiarity of response seen in the esophagus. Moreover, in the second study, EGF treatment had no effect on hepatic DNA synthesis in the fasted female mice.

Pancreas

Figure 3 depicts a typical pattern of response to EGF in DNA synthesis in the pancreas. Fasting in both sexes decreased the overall level of DNA synthesis. An unexpected finding was that DNA synthesis in the EGF-treated mice began to increase as late as 23 hr after the initial injection of EGF and remained elevated compared to controls to the end of the study. The increases seen at 23, 28, 33, and 38 hr after the initial injection of EGF were 73%, 59%, 77%, and 103%, respectively ($p<0.01$). There was a significant ($p<0.05$) increase in DNA synthesis in the EGF-treated mice compared to their saline-treated counterparts over the 30 hr sampling span of 50%. EGF had no effect on DNA synthesis in the pancreas of male or female fasted animals, although in light of the data reported herein on ad lib mice, an organ response might have been seen at later times; additional studies are indicated.

Kidney

In the EGF-treated mice, statistically significant increases in DNA synthesis of 55%, 50%, and 58% occurred at 23, 28, and 33 hours, respectively. There was an overall increase of ($p<0.05$) 25% for the 30 hour sampling time. In the fasted male animals, a significant ($p<0.001$) increase in

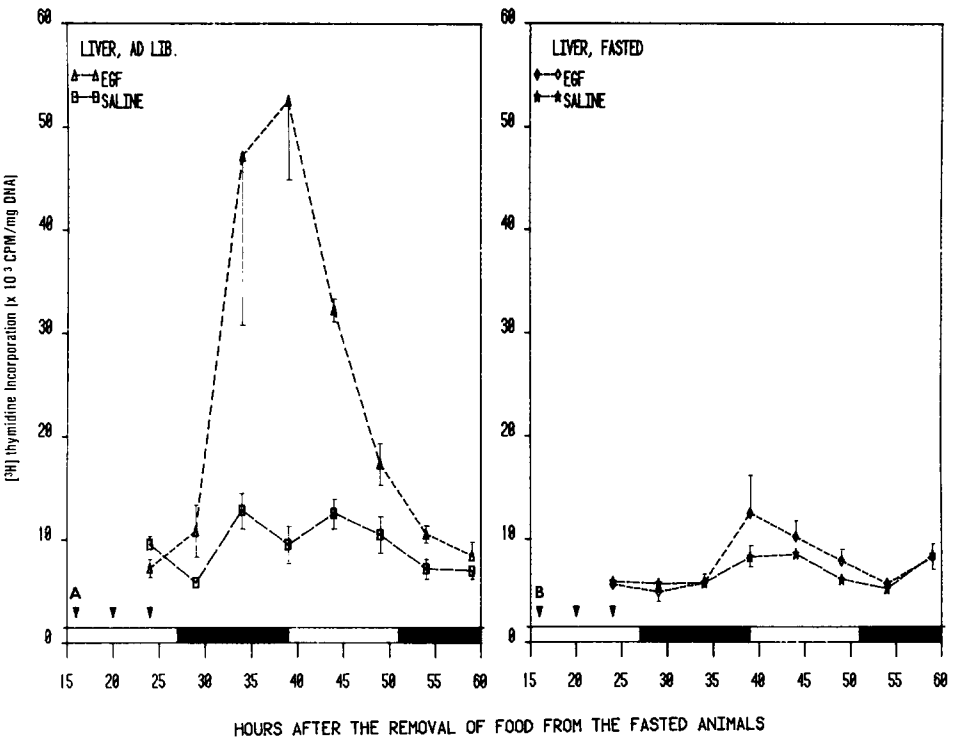

FIG. 5. [^{3}H]-thymidine incorporation into DNA in the liver of in ad lib fed and fasted CD2F1 female mice. EGF did not stimulate DNA synthesis in fasted mice, but had a dramatic effect in ad lib fed mice. See legend in Fig. 1 for a more detailed explanation.

DNA synthesis (36%) was noted only at the 8 hour sampling time (Fig. 6A). No significant differences in synthesis rates were recorded between EGF and saline-treated fasted females (Fig. 6B).

DISCUSSION

In this study, we have examined the effects of exogenous EGF on DNA synthesis in four different organs of male and female ad lib fed and fasting mice—the esophagus, liver, kidney, and pancreas. Additionally, we have studied in the esophagus and liver the effect of EGF in more detail in fasted mice. Our work demonstrates that although EGF stimulates DNA synthesis in each organ, the time course and magnitude of response vary. For example, EGF stimulated DNA synthesis most rapidly and to a greater degree in the esophagus; however, because synthesis rates dropped below normal at later times, the average level over the entire experimental time frame was not significantly different when compared with controls. In contrast, in the other organs, the time-averaged level of DNA synthesis was greater in the EGF-treated mice compared to the controls even though the stimulatory effects occurred later and were of smaller magnitude. These differences probably represent an intrinsic differential organ response mediated by the interaction of EGF with specific receptors; however, we cannot exclude the possibility that they are related to effects of other hormones possibly secreted in response to EGF, such as prolactin, luteinizing hormone, and growth hormone [9, 10, 14, 20]. In any event, our data do show how limited sampling times could lead to erroneous conclusions concerning the response of individual organs to EGF. For example, we have previously been unable to show that EGF stimulated DNA synthesis in the pancreas because we sampled at less than 16 hours after injection.

We have previously established that the organs studied in this paper exhibit circadian variation in DNA synthesis and mitotic index. Of these, the esophagus shows the highest amplitude of circadian variation, peaking in late dark phase and returning to trough levels in the middle of the light period. Drawing on parallels to *in vitro* work, we originally proposed that the circadian rhythms in cell proliferation might be the result of changes in the local or systemic release of various growth factors such as EGF, PDGF, or interleukin II, or in changes in the biochemical properties or numbers of their cell surface receptors [16–17, 22].

Although we had shown in previous studies that EGF could stimulate DNA synthesis in the esophagus and other organs within 16 hours after injection, we did not know to what extent it would affect the normal circadian rhythm of DNA synthesis in the esophagus. Therefore, in this study, we injected EGF during the light phase and studied its effect over a longer time frame. We injected EGF during the light phase because this is the time when the levels of esophageal DNA synthesis as well as circulating [15] and salivary [12] EGF are lowest. Under the conditions of this study we found that the normal circadian rhythm did not manifest itself. Rather, exogenous EGF treatment phase-shifted the rhythm so that the peak occurred at the transition from light to dark, rather than late in the dark phase (Fig. 1).

Because fasting has been shown by us to ablate or at least alter the cell proliferation rhythms in EGF target organs of mice [19], we anticipated that it might affect the organ response to EGF. Thus, in this study we examined the effect of EGF on DNA synthesis in the esophagus and liver of fasted

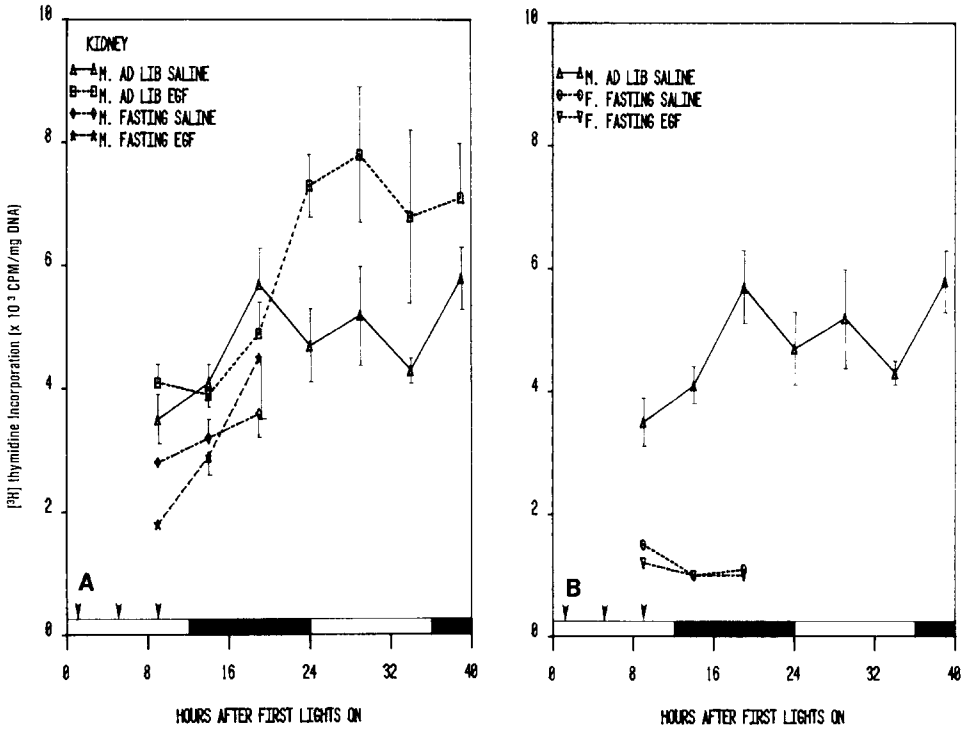

FIG. 6. Data obtained in the kidney. Panel construction as in Fig. 1.

mice. Confirming previous studies, fasting clearly reduced but did not eliminate circadian variation in both organs. In this respect, both target organs behaved similarly. However, their responses to exogenous EGF in the fasted animal were remarkably different. In the esophagus, the stimulatory effect was augmented and prolonged compared to ad lib fed controls. In contrast, in the liver, the effect of EGF in the fasted mice was virtually abolished. The mechanisms by which these changes occur are unknown; however, they may involve changes in various biochemical properties of the EGF receptor in the two organs. In addition, the observation that the cellular concentration of various glycolytic metabolites can affect the growth response of cultured hepatocytes to EGF may be related to the decreased response of the liver [13].

Because there are sex differences in the circadian rhythms of submandibular gland EGF synthesis [6], serum EGF levels [15], hepatic DNA synthesis [21] and the hepatic EGF receptor [3], we expected that there would be sex differences in response to EGF. Under the conditions of the first study, which used fasted mice killed within a rather short time frame, we found no obvious differences between sexes. However, a comparison of the results of the first and second studies, the first of which included male fed mice and the second female fed mice, reveals what appears to be an interesting sex difference in the response of the liver and esophagus. The response of the liver in the female mice (Fig. 5) compared to the males (Fig. 4) appears to be more rapid, in contrast to the esophagus, where no such difference was seen. Obviously, further studies are required to address this point comparing ad lib fed male and female mice directly under identical conditions at the same time.

In this study we have shown that EGF can alter the normal circadian patterns of DNA synthesis in the esophagus. This ability could prove useful in the treatment of various forms of cancer. Although the current pathological classification of tumors is primarily morphological, it may become possible to classify tumors based on biochemical or genetic parameters involving growth factor expression. This would enable the oncologist or radiologist to administer to the patient specific growth factors to synchronize normal cell populations but not tumor cell populations. By judiciously timing this synchronization with respect to the tumor treatment, one should be able to decrease toxicity to normal cell populations without affecting tumor toxicity [6,19].

Finally, we have presented in this paper additional data documenting that fasting dramatically affects DNA synthesis in a number of organs. This fact may also be clinically relevant with respect to the administration of chemotherapy or radiotherapy [19].

ACKNOWLEDGEMENTS

Supported in part by grant OH-00952 from the National Institute of Occupational Safety and Health.

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