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EFFECT OF AN ADRENOCORTICOTROPIN ANALOGUE, ACTH 1-17, ON DNA SYNTHESIS IN MURINE METAPHYSEAL BONE*

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Abstract—The effects of injections of a synthetic adrenocorticotropin (ACTH 1-17, Synchrodyn) on the rate of DNA labeling in the metaphyseal bone of $CD2F_1$ mice were tested on a chronopharmacological dosing schedule. Groups of mice that had been conditioned to a 12-hr light/12-hr dark schedule were injected at one of six different timepoints, 4 hr apart, during a single 24-hr span with either a low (0.02 I.U./kg) or a high (20 I.U./kg) dose of ACTH 1-17. Control groups received injections of a placebo at corresponding timepoints. Subgroups of mice were injected with [^{3}H]thymidine ([^{3}H]Tdr) to follow the changes in DNA labeling in the proximal tibial metaphysis at 15 min and 2, 4, 8, 12 and 24 hr after ACTH 1-17 or placebo treatment. All mice were injected with the isotope 30 min before killing, except for those killed 15 min after R_x administration where the isotope had been injected 14 min before killing. The data were analyzed both by analysis of variance and by the cosinor method, the latter of which tests the fit of a 24-hr cosine curve to the data. The effect of ACTH 1-17 on the target cell population was dependent not only upon the dose but upon the time of administration. Both doses exerted time-dependent action, ranging from stimulation to inhibition of DNA labeling. Inhibition was noted when the ACTH 1-17 was administered at 2 hr after the beginning of the daily dark span when nocturnal animals become active. When administered at this circadian stage, the larger dose in particular was associated with an inhibition of DNA labeling lasting for 24 hr. The inhibitory effect was much shorter when the same dose was injected 4 hr earlier. Moreover, the large ACTH 1-17 dose had a stimulatory effect lasting for 24 hr when it was administered 2 hr after the onset of the daily light span, with a much shorter stimulation following administration of the large dose at 6 hr after the beginning of the daily dark span. A circadian stage-dependent stimulation or inhibition of DNA labeling at 2 or 14 hr after light onset, respectively, was thus complemented by an initial inhibition followed by stimulation and vice versa at 10 and 18 hr after light onset respectively. As a whole, the circadian stage-dependent effects of ACTH 1-17 injections on murine metaphyseal DNA labeling revealed a unified biologically and statistically significant relationship among changes in DNA labeling rate and the host state at treatment time, the kind and dose of treatment, and the time elapsed from injection to killing. Such relationships need not be viewed as confusing variability. Instead, they may be exploited as a unified feature of pharmacodynamic interaction, as chronomodulation. Further chronobiologic designs and analyses will have to examine the question whether the time-dependent ACTH 1-17 effects at the cellular level are exerted via corticoids at certain times and via other hormonal or direct action at other times.

In attempts to reproduce in laboratory animals the spontaneous bone fractures due to ostoporosis seen in Cushing's Syndrome and after prolonged treatment of patients with glucocorticoids, Vogel in 1969 "... found unexpected results" [1]. Within a few hours after single injections of glucocorticoid, the strength of all connective tissue in all species tested by him was increased markedly. Vogel described a "biphasic" effect of glucorticoids upon the tensile strength of the epiphyseal–metaphyseal apparatus, tendons and skin strips which pertained to the breaking strength of bones; low and medium-high doses of cortisol increased breaking strength while high (toxic) doses decreased it. Variability in the effects of ACTH on bone growth and remodeling, though less extensively studied, has been similarly interpreted as primarily an effect of differences in dosage [2–6].

In rat mandible and femur, steroids affect appositional bone growth by a circadian stage-dependent mechanism. The effects of a short-acting steroid such as methylprednisolone sodium succinate (MPSS) differ depending not only upon the dose and frequency of injections, but also upon the circadian stage of drug delivery defined in relation to the lighting regimen used to standardize the rats [7–9]. On an every day (ED) or alternate day (AD) schedule, sup-

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pressive effects of the steroid on bone formation were expressed most readily when it was administered in the middle of the dark span (D) in animals kept in light (L) and D alterating at 12-hr intervals (LD 12:12). Treatment on an AD schedule (dose = 2 ED) increased the rate of bone formation when MPSS was administered early in the daily light span. An ED schedule also increased distal femoral metaphyseal trabecular density in those animals treated with MPSS early in the daily light span.

Circadian time-dependent effects on articular cartilages of rats following chronic intra-articular injections of a long-acting steroid such as cortisone acetate have also been reported [10, 11]. In each of these studies, the stimulating or suppressive effects of the steroids were compatible with a circadian pattern of matrix synthesis.

In this study, we investigate, as a function of timing, the action of ACTH 1-17 (HOE 433; Synchrodyn) on DNA labeling in the metaphyseal bone of mice. This ACTH analogue has documented time-dependent effects in human beings and rodents. In human beings, its administration is compatible with a circadian pattern of serum and urinary cortisol and of urinary calcium, phosphorus and electrolyte excretion. The analogue also has high corticosteroidogenic potency, with the consequence of demonstrated effectiveness when administered to man intranasally, an attractive mode for clinical administration [12–23].

MATERIALS AND METHODS

In December 1979, $CD2F_1$ mice, approximately 6 weeks of age, were received from the Simonsen Laboratories, California, weighed and randomly assigned seven animals/cage. Four to five cages (twenty-eight or thirty-five mice) were then randomly placed in individual isolation chambers, each controlled for light and temperature. Fluorescent illumination within the chambers was programmed so that half the mice were exposed to light from 0600 to 1800 (CST: LD environment) daily and half were in light from 1800 to 0600 (CST: DL environment). Food and tap water were available *ad lib*. Cages were cleaned once a week on the same day. The reversed lighting regimen was instituted for over 3 weeks so that the circadian studies could be performed during regular laboratory working hours. It was assumed, for instance, that at 0800 CST, the circadian system of mice conditioned in the DL environmental chambers would correspond to that of mice in LD at the 2000 point, i.e. at 2 hr after the beginning of their active span. The animals were kept in this regimen for 31 days. When the study began, the animals weighed 20 ± 2 g (mean ± S.E.).

The study followed the time course of the effect of two dose levels of a synthetic short-chain adrenocorticotropin, ACTH 1-17, on metaphyseal bone DNA synthesis. We examined the responses from 15 min to 24 hr following administration at six different time points (4 hr apart) in a 24-hr day. Thus, by injecting different groups of mice in the chambers with ACTH 1-17 at 0800, 1200 and 1600, the mice in LD received the steroid at either 1, 6, or 10 hr after light onset (HALO) and those in DL at either 14, 18, or 22 HALO. At each of these circadian stages, forty-two mice distributed in six cages were injected subcutaneously (s.c.) with 20 I.U./kg ACTH 1-17; forty-two mice were injected with 0.02 I.U./kg ACTH 1-17; and forty-two mice were injected with a placebo. The injection volume was 0.2 ml/20 g mouse body weight.

Seven animals from each of the ACTH 1-17 and placebo-injected groups (0800, 1200, 1600, 2000, 2400 and 0400) were killed by cervical dislocation at 15 min, 2, 4, 8, 12, and 24 hr after treatment. Each animal (except those in the group killed 15 min after injection) also received an intraperitoneal (i.p.) injection of 25 μCi tritiated thymidine ([^{3}H]Tdr: New England Nuclear Corp., Boston, MA; sp. act. = 25 Ci/mmole) 30 min before killing. The groups of mice that were scheduled to be killed 15 min after treatment were injected with [^{3}H]Tdr immediately after receipt of the hormone or placebo.

At autopsy, the tibias were recovered and fixed in 10% neutral formalin. The bones were then split longitudinally, and the proximal metaphyses containing trabecular bone were dissected free from the cortices and epiphyseal cartilages. The DNA from this relatively marrow-free area (see below) was extracted in 1 N NaOH, and concentrations of DNA were quantified according to the indole reaction method of Keck [24] following trichloroacetic acid (TCA) precipitation of the protein to eliminate acid-soluble pools. Previously, we did not find that radiolabeled thymidine acid-soluble pools vary predictably during a 24-hr study span [25; cf. also Ref. 26]. An aliquot of the DNA extract was neutralized with 6 N HCl and used to quantify the radioactivity incorporated into the DNA. The radioactivity was counted in a Packard Tri-Carb liquid scintillation counter. All samples were corrected for quenching, and the data were expressed as disintegrations per min (dpm) ^{3}H/mg DNA.

We are dealing herein with a mixed population of cells—hemopoietic marrow cells comprise perhaps 20% of the total metaphyseal cell population. However, the measurements of DNA labeling, as an approximation of synthesis, largely reflect the activities of the osteoprogenitor cells which give rise to the mitotically inactive osteoblasts and osteocytes. The DNA rhythms of metaphyseal cells and diaphyseal marrow, however, are quite similar. Formalin fixation will extract some DNA from the tissues [26], but such extracts contain a uniform ratio of labeled/unlabeled DNA.

The data were subjected to analyses of variance (ANOVA), complemented by cosinor methods and regression diagnostic tests [27–29]. Single cosinor analyses were performed on separate data sets from the groups of mice treated at different circadian stages, but killed at each of the intervals after treatment.

RESULTS

The results at injection times which were documented by nearly complete data are shown in Table 1 as means ± S.D., with an indication of lost samples. The effects include both overall inhibition and stimulation of DNA synthesis. Figure 1 shows that the

Table 1. Metaphysical bone DNA labeling*

R_x time (HALO)†	DNA synthesis (dpm/mg DNA)		
	Placebo	ACTH 1-17 0.02 I.U./kg	ACTH 1-17 20 I.U./kg
2	55.12 ± 30.89 (15)	55.79 ± 49.28 (26)	134.90 ± 171.21 (33)
6		14.90 ± 6.97 (37)	16.27 ± 14.03 (40)
10	121.30 ± 67.48 (12)	101.03 ± 57.23 (33)	103.95 ± 134.70 (35)
14	62.38 ± 29.97 (22)	45.23 ± 23.16 (37)	16.36 ± 8.59 (32)
18	45.62 ± 31.32 (19)	13.47 ± 10.40 (37)	62.58 ± 49.59 (28)
22	52.97 ± 9.65 (17)	26.72 ± 26.73 (34)	30.43 ± 12.37 (26)

* Values are means ± S.D.; the number of observations is given in parentheses.
† HALO = hours after light onset.

effects of the high dose of ACTH 1-17 were qualitatively as well as quantitatively dependent upon circadian timing. For the 24 hr after ACTH 1-17 administration, metaphyseal DNA labeling appeared to be stimulated at one time and inhibited at another. At other injection times, stimulation of DNA synthesis was either followed by inhibition, or inhibition was followed by stimulation. These different effects are in keeping with the results from a three-way ANOVA (Table 2).

Two separate approaches suggested that the ACTH 1-17 time–response structure was altered but not obliterated. One particular line of evidence is based upon single P values < 0.05 (data not shown) resulting from the cosinor approach to data as a function of different injection times at each fixed killing time. The likelihood by a non-parametric sign test that the circadian structure was lost—as far as it can be approximated by a cosine fit—is extremely small ($P < 0.01$). The rhythm characteristics derived

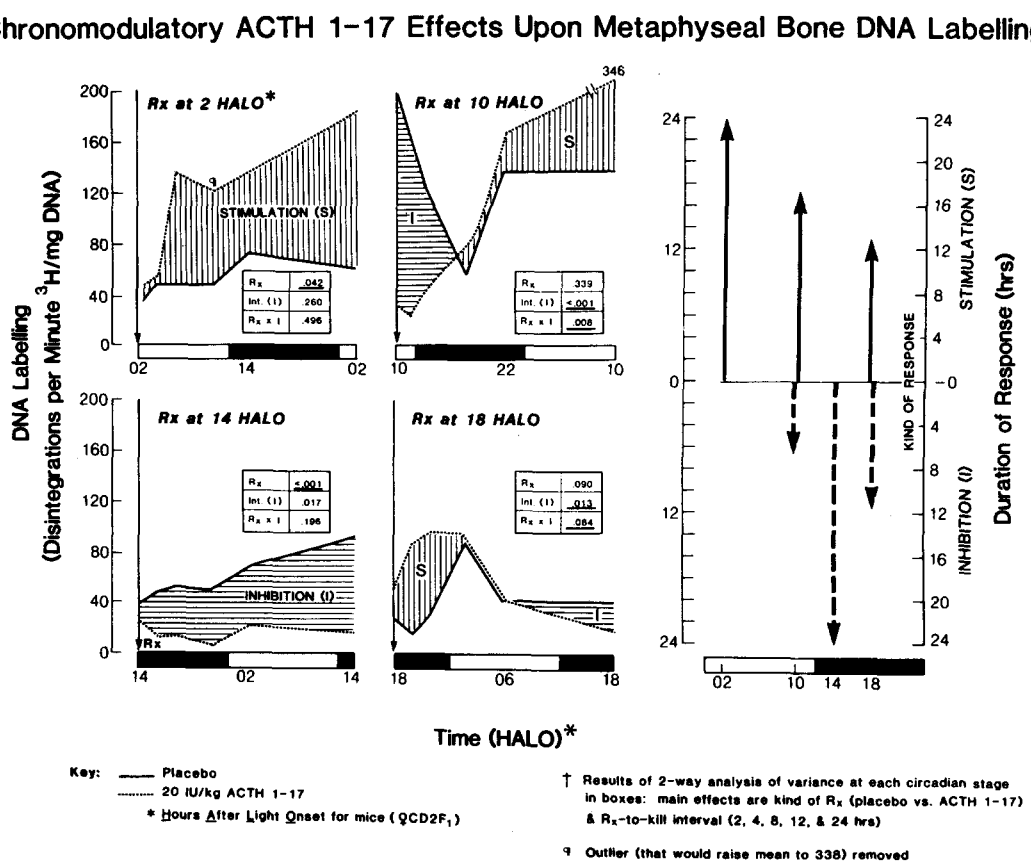

Fig. 1. DNA labeling in $CD2F_1$ mice during 24 hr after treatment by placebo or ACTH 1-17 (left) and duration of response, based on the same data (right). The statistical significance of the findings was established by three-way ANOVA of all data (see Table 2) and by two-way ANOVA for data at each treatment time. The latter yielded the P values inserted in the four left-hand sections of the graph for the effect of treatment (R_x), those of the time interval elapsed since treatment (Int) and for interaction.

Table 2. Effects of ACTH 1-17 on metaphyseal bone DNA labeling

Source of variation	DF	F	F
(A) All data			
Main effects			
R_x kind (placebo, low or high dose ACTH 1-17)	2	6.25	0.002
R_x time (2-, 6-, 10-, 14-, 18- or 22-HALO)	4	24.85	0.001
R_x-to-kill interval (15 min, 2, 4, 8, 12, or 24 hr)	5	4.40	0.001
Two-way interactions			
R_x kind vs R_x time	8	5.72	0.001
R_x kind vs R_x-to-kill interval	10	2.92	0.002
R_x time vs R_x-to-kill interval	20	3.24	0.001
Three-way interaction			
R_x kind vs R_x time vs R_x-to-kill interval	32	3.35	0.001
(B) Comparison between effects of placebo and high dose of ACTH 1-17 (20 I.U./kg)			
Main effects*			
R_x kind	1	1.04	0.309
HALO time†	3	10.49	0.001
R_x-to-kill interval	4	4.05	0.004
Two-way interactions			
R_x kind vs HALO time	3	5.00	0.003
R_x kind vs R_x-to-kill interval	4	1.15	0.335
HALO time vs R_x-to-kill interval	12	3.11	0.001
Three-way interaction			
R_x kind vs HALO time vs R_x-to-kill interval	12	2.03	0.026

* R_x kind: placebo vs high dose of ACTH 1-17 (20 I.U./kg).
HALO time: 2, 10, 14, 18.
R_x-to-kill interval: 2 hr, 4 hr, 8 hr, 12 hr, and 24 hr. Data from R_x-to-kill interval of 15 min was omitted since missing from the placebo group at 10-HALO; at 2-HALO, the DNA labeling value was interpolated for the placebo group at 2 hr after R_x (the adjacent mean values and S.E. were 50.4 ± 2.6 and 49.4 ± 9.6).

† HALO = hours after light onset.

by cosinor, measures of overall mean and extent and timing of change, however, clearly do not show consistent values. Regression diagnostic tests, carried out to check on sinusoidality, homogeneity of variance and normality of residuals—assumptions which underlie the cosinor—are violated in a number of cases. However, for all cases, the conclusions concerning different circadian stage-dependent effects are supported by three-way analyses of variance (Table 1) and by the results of separate two-way analyses (see inserts in Fig. 1).

The control data were incomplete at several time points after administration of the placebo. All placebo groups were missing after ACTH 1-17 injection at 6-HALO; also missing were the 8- to 24-hr post-injection data at 22-HALO and the 15-min to 2-hr post-injection data at 10-HALO. The data points available indicate nonetheless that the placebo injections were not without their own circadian stage-dependent effects. Section A in Table 2 reports the results of a three-way analysis of variance on all data, and shows that all main effects played a role and that their effects were all interacting.

To gain a better understanding of the interactive effects, a second three-way ANOVA was carried out by considering only the placebo and high dose ACTH 1-17 (= Placebo R_x kind) at 2-, 10-, 14- and 18-HALO with R_x-to-kill intervals from 2 to 24 hr (Table 2, Section B). This analysis avoided the difficulties of interpretation due to missing data points. Again, the treatment time and R_x-to-kill interval were statistically significant main effects. Although no R_x-kind effect is demonstrated here, this effect interacts with statistical significance with R_x time. To visualize this interactive effect, separate two-way ANOVA were performed at each separate R_x time. The results are illustrated in Fig. 1. It can readily be seen that the ACTH 1-17 effects varied as a function of R_x time. Following treatment at 2-HALO, the mean values for metaphyseal bone DNA synthesis in the mice treated with the large dose ACTH 1-17 were consistently greater than those recorded in the mice treated with the placebo. It was of interest that, at this injection time, the small dose of ACTH 1-17 was associated with a large increase in DNA labeling 2 hr after injection, followed by a decrease to (or slightly below) placebo-like values, and there were no differences between these groups at 24 hr (data not shown). With treatment at 10-HALO, the large dose of ACTH 1-17 inhibited DNA synthesis at 4 hr after injection, but the effect was transitory. With treatment at 14-HALO, the large dose of ACTH 1-17 caused a prompt and pronounced decrease in DNA synthesis by 15 min; this inhibition persisted for the full 24 hr of the study. With treatment at 18-HALO, the large dose of ACTH 1-17 stimulated DNA synthesis during the first 4 hr of the study but decreased it slightly by 24 hr post-injection. Thus, these complex and seemingly contradictory results may, in fact, be the consequence of complex chronomodulatory effects, called a "feed sideward effect" [19–21].

DISCUSSION

Circadian stage-dependent differences in responsivity and/or lack of responsivity—and the differences in the rapidity of onset of the effects on DNA synthesis—can be a powerful tool by which to manipulate bone cell metabolism. Companion papers detail the equally important effects of chronopharmacologic ACTH 1-17 actions on the mitotic index of corneal epithelium [18] and upon the development of a circadian rhythm of tolerance to doxorubicin in mice [12, 17]. We cannot be certain whether ACTH 1-17 has any effect upon the metabolism of bone cells which is not secondary to stimulation of adrenal corticosteroid production [25, 30].

There exist certain parallels between the results reported in this study and our previous experience with the chronopharmacologic actions of a short-acting synthetic steroid on linear and radial bone growth processes in rats [MPSS: 7–9]. In the MPSS Study, daily injections in excess of 1.0 mg/kg most significantly inhibited bone growth/mineralization when the steroid was administered during the environmental dark span (tetracycline-based data). In those animals treated for 6 days with either 0.25 mg/kg/day or with 0.5 mg/kg/every other day, bone growth/mineralization was selectively stimulated by injections which occurred early in the daily light span (1-HALO). Other data obtained in the MPSS study were related to the chronopharmacologic effect of the steroid on bone remodelling in the distal femoral metaphysis. At the end of a 46-day treatment course with daily injections, the density of metaphyseal trabeculae was increased significantly in those animals treated at the middle of the daily light span and decreased in those groups treated at the middle of the daily dark span. In meal-timing studies, we have also shown that the extent of DNA synthesis in epiphyseal cartilage, metaphyseal bone and diaphyseal bone varies in a seeming direct relation with the circadian changes in serum corticosterone [30], and that the rhythms can be abolished by adrenalectomy [40]. Exposure of osteoblast cultures to glucocorticoids has resulted in a "biphasic" response, i.e. stimulation of Type I collagen formation and alkaline phosphatase values after 24 hr, but diminished values of both measured after 96 hr [31]; however, our meal-timing studies have not shown a relationship between the usual circadian rhythms in serum corticosterone and collagen formation in any region of the rat tibia [30]. There is, then, reason to consider that most if not all of the changes in metaphyseal DNA synthetic rates reported herein are secondary to ACTH-generated adrenal corticosteroid production, even when the large ACTH dose depressed metaphyseal DNA synthesis as rapidly as 15 min (14-HALO, 2000 hr). The reciprocal or near-reciprocal relationship between the ultradian secretions of ACTH and adrenal corticosteroids may have been particularly well tuned at this stage of the circadian cycle [32, 33]. Moreover, it is well known, particularly from *in vitro* organ cultures, that the responsivity of the adrenal to ACTH is circadian in nature [34–38].

Many neuroendocrine interactions are characterized by having intrinsically periodic components, and they may be influenced by cyclic external factors, each of which has a spectrum of rhythms. The need to account for interrelations among the pineal, hypothalamus, pituitary, and adrenal prompts us to replace the term *axes* with *networks*, since any one of these entities can interact with any other and does so *in vitro* [38]. The recognition of interwoven, interdigitating links in networks of so-called feed-sideward effects prompts the search and scrutiny within the networks of corresponding punctual modulations. The feed-sidewards of networks result in coordinate rhythmic interactions by one entity (the modulator) with the interaction of two other entities (the actor and the reactor), some or all of them intrinsically periodic. Moreover, an actor in one such functional triangle can assume the role of modulator in another triangle. The pineal modulation of the pituitary interaction with the adrenal (*in vitro*) is a case in point [21]. The pituitary modulation of adrenal interactions at the level of bone DNA synthesis reported herein may well be another example. For two of the cited entities, the adrenal and the pineal, a bioperiodicity has been demonstrated in organ culture. It seems likely, but not essential, that an intrinsic periodic component should be present in all participants of a feed-sideward. In all of them, there are likely additional interactions, notably when feed-sidewards involve the neuroendocrines. Feed-sidewards can be found in all networks and kinds of biologic organization, and they represent new biochemical endpoints for students of the pharmacodynamics of modulation.

With reference to the central nervous system, i.e. in a different context, Otto H. Schmitt has referred to interpenetrating domain representations as an approach which can assign one element of a system, such as a neuron, as part of several complexes simultaneously. Each complex utilizes the same element but in a variety of roles, often conflicting in character but operating overall in a consistent manner. He refers to interpenetrating domain analysis as a form of bookkeeping which allows one to pursue the individual information processes in metabolic, mechanical, neuroanatomical, and behavioral games that are going on individually within a neuron system . . . allowing one to consider them interactively when the need arises.*

The interdigitating network approach of the chronophysiologist is a similar endeavor in complex bookkeeping within three webs of rhythms: neural, hormonal and cellular [39]. Such network theory is useful if and only if it can sharpen the focus on the interpretation of actual data bearing upon specific problems. The effect of ACTH on bone may be a pertinent interesting problem. Our data provide a further demonstration that experimental design must be sensitive to the temporal organization of the physiology of experimental animals and, as Vogel found

* O. H. Schmitt, *Interpenetrating domain representation for information processing and control in neuronal assemblies*; invited presentation, May 16, 1972, to MIT Neuroscience Research Program, Work Session on Dynamic Patterns in Brain Cells, May 14–16, 1972, F. O. Schmitt, foundation scientist; Aharon Katchalsky and Vernon Rowland, co-chairmen. Available as a manuscript.

[1], results may be unexpected until timing is wedded to dosing.

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