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Theoretical basis of Microdose Therapy

Negative endocrine control system for inflammation in rats

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Abstract

Inflammatory processes may be suppressed by endogenous mechanisms such as release of adrenocorticosteroid hormones through stimulation of the hypothalamus-pituitary-adrenal axis. In the present study, the relationship between the temporal development of carrageenan-induced edema in the hind limb of the rat and release in plasma of the principal endogenous adrenocorticosteroid of the rat corticosterone was investigated. Suplantar injection of carrageenan produced a biphasic increase in basal plasma corticosterone levels that was not attributed to diurnal variation. The plasma level of corticosterone increased rapidly after injection of carrageenan and peaked 12-fold at 20 min. This first phase increase was attributed to the stress of the injection since it was mimicked by subplantar injection of saline. The second phase of corticosterone release was gradual and peaked 12-fold 7 hr after injection of carrageenan. The second phase was not elicited by subplantar injection of saline. When the hypothalamus-pituitary-adrenal axis is impaired via hypophysectomy, carrageenan-induced edema is more intense and last longer than in control rats. The results demonstrate that adrenocorticosteroid hormones are released as a result of the stress of injection and by the inflammatory response. Release of adrenocorticosteroids acts as a feedback mechanism to suppress the inflammatory response.

Introduction

If inflammation could be demonstrated to cause a pulsed adrenocorticosteroid hormone release, a case can be made for the existence of a negative endocrine control system for inflammation. Should such pulsed steroid release occur, its intensity and time of release would be important in order to effectively first allow then limit the duration of inflammation.

Currently, the stress-stimulated hypothalamus is believed to secrete corticotropin-releasing hormone. This hormone activates the neighboring anterior pituitary to release adrenocorticotrophic hormone. The latter hormone is transported to the adrenal cortex and stimulates it to discharge a variety of steroids. The steroid release from the adrenal cortex influences the hypothalamus/anterior pituitary to reduce the adrenocorticotrophic hormone output via a negative feedback control system. Overall, this is defined as the hypothalamus-pituitary-adrenal axis. Of the steroid released from the adrenal cortex, the glucocorticoid subgroup has a most powerful controlling effect on inflammation [1]. Leme et al. [2,3] first proposed that a regulatory system exists in which the hypothalamus is activated by acute inflammatory responses. Insulin, glucagon and corticosteroids were

designated as inflammatory, indirect anti-inflammatory, and anti-inflammatory agents, respectively.

This investigation evaluates (1) the ability of inflammation to cause a pulsed adrenocorticosteroid hormone release [4-6] in the plasma of rats, (2) the relative concentration intensity of the steroid release, and (3) the time relationship of the inflammation with the steroid release. The experiments couple the carrageenan-induced rat paw edema model for acute inflammation with plasma analysis for the principal adrenocorticosteroid released in the rat, corticosterone.

Materials and methods

Animals and diets

Adult, male Sprague-Dawley rats, which weighed 200-300 g at the time of experiment, were purchased from Bio-Lab (Minneapolis, MN). The hypophysectomy surgery was done by the company supplying the rats. The rats were group housed (4 per cage) in stainless steel cages having 157 square inches of floor space. The ad lib diet of Purina Rodent Laboratory Chow 5001 was purchased from Ralston Purina Co. (St. Louis, MO). Tap water was continuously available. The temperature of the animal room was maintained at 22 \pm 1°C, and the relative humidity was kept at 50 \pm 0.20%. There was a fresh air exchange rate of 15-18 changes per hour. The animal room has a 12-hour light/dark cycle. The light cycle began at 7:00 a.m. Heparin sodium (140 USP Units per mg) was purchased from Sigma Chemical Company (St. Louis, MO). Carrageenan was donated by FMC Corporation, Marine Colloids Division (Rockland, MA), reference number 9259. The corticosterone assay kit was purchased from Radioassay Systems Laboratories (Carson, CA). The edema measurements were done in the conventional manner.

The rat hind paw volume was measured on anaesthetized animals by a water displacement method [7].

Injection of Carrageenan

The day before each experiment, 14 rats were transferred into stainless steel cages with 68 square inches of floor space (2 per cage), brought to the procedure room, weighed and allowed overnight to acclimate to the conditions. Six rats were injected with 0.05 ml of a 1% carrageenan suspension in a 0.9% saline solution through a 26-gauge needle into the foot pad of the right hind paw [8]. Another six rats were injected in the same manner with 0.9% saline solution without carrageenan. The remaining two rats received no injection, and these served as controls for the determination of the basal plasma level of corticosterone. The carrageenan injections were done in a timely fashion in order that the blood samples were collected between 11:00 a.m. and 12:00 noon. In this manner, the diurnal variation corticosterone increase of the afternoon would not complicate the data.

Blood Sampling

The rats were placed under ether anesthesia in order to obtain plasma samples except for those samples obtained at 10, 20, 40 and 50 minutes. For the 10-, 20-, 40- and 50-minute samples, 0.1 mg/100 g injections of sodium pentobarbital were used as the anesthetic.

The blood samples were obtained by cardiac puncture with a heparinized needle and syringe. The samples were transferred to 10x75 mm heparinized test tubes and centrifuged. The plasma was separated and frozen until analyzed for corticosterone. All blood samples were obtained within 2.0 minutes after the animal was removed from its cage. The plasma of each rat was individually analyzed for corticosterone content.

Radioimmunoassay

After equilibration at 98°C for 10 minutes and subsequent cooling to room temperature, the plasma samples were reacted with the antibody solution. Next, the corticosterone-3H tracer containing ca. 10,000 cpm/0.1 ml was added to the samples. Thereafter, samples were equilibrated for 8 hours in a 4°C temperature bath. After incubation, the samples were treated with charcoal at 4°C for 20 minutes. After centrifugation at 2500 rpm for 15 minutes, the mixture was decanted into the scintillation cocktail. The scintillation cocktail samples were counted in a Packard Tri-Carb Scintillation Spectrophotometer Model 2003. The scintillation cocktail, Insta-Gel, was purchased from Packard Instrument Co., Inc. (Downers Grove, IL). The procedure measured the sum of unbound and bound corticosterone.

Statistical analysis of results

Table 1 illustrates the data together with the results of the error analyses. The errors are calculated using standard deviation of the mean.

Results

When nonstressed Sprague-Dawley rats are injected with 0.05 ml of a 1% saline solution of carrageenan, two concentration pulses of corticosterone are observed in the blood plasma, cf. Table 1. The first pulse is a sharp one which has a maximum at 20 minutes after injection. The second pulse is broad and begins at 5 hours after injection. The latter pulse maximizes at 7 hours after injection. The latter pulse maximizes at 7 hours and terminates 10 hours after injection. A subplantar injection of saline produced the first pulse but not the second broad pulse, cf. **Figure 1**. Both pulses exhibit 12-fold increases over the basal plasma level of corticosterone.

Table 1 Results and statistical analyses on rat experiments.

Time, min.	Control, no. rats	Saline, no. rats	Carrageenan no. rats	Corticosterone, ng/ml ^a
0	18	-	-	25 +/- 9 ^b
20	2	-	-	28
60	2	-	-	51 +/- 9
120	2	-	-	28 +/- 9
150	4	-	-	22 +/- 9
240	2	-	-	16 +/- 9
300	2	-	-	16 +/- 9
420	2	-	-	58 +/- 9
600	2	-	-	2 +/- 9
20	-	9	-	340 +/- 30 ^c
20	-	4	-	336 +/- 10
60	-	4	-	42 +/- 24
120	-	5	-	40 +/- 17
180	-	5	-	25 +/- 26
240	-	5	-	16 +/- 5
300	-	7	-	8 +/- 6
420	-	6	-	24 +/- 19
600	-	5	-	3 +/- 1
10	-	-	4	183 +/- 27 ^c
20	-	-	3	308 +/- 16 ^c
20	-	-	5	267 +/- 67
30	-	-	3	285 +/- 47 ^c
40	-	-	4	212 +/- 66 ^c
50	-	-	4	106 +/- 6
60	-	-	6	44 +/- 31
120	-	-	5	21 +/- 7
180	-	-	5	12 +/- 3
240	-	-	5	26 +/- 10
300	-	-	12	145 +/- 70
420	-	-	6	292 +/- 72
600	-	-	5	12 +/- 10

a 90% confidence.

b The mean of the following 8 entries of this Table.

c Sodium pentobarbitol anesthesia.

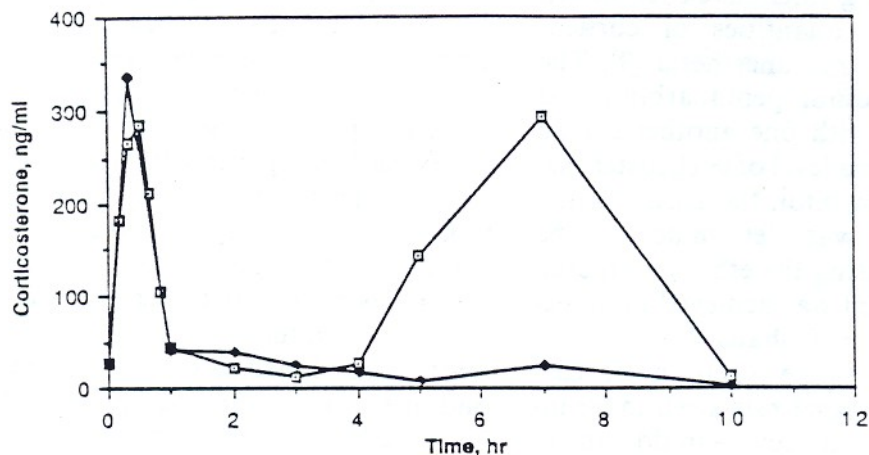


Figure 1
Plasma corticosterone response from carrageenan (□) and saline (◆) injections.

To evaluate physical handling stress of the rats during the experiments, one control group of rats was handled identical to the rest except they were neither injected with carrageenan nor saline solution. This group showed no statistically significant increases of corticosterone as a function of time. Therefore, the physical handling of the rats as utilized in the course of the experiment was not responsible for the observed pulses of corticosterone. The blood samples obtained within the first two minutes after administering ether have been shown to contain insignificant quantities of corticosterone induced by the ether anesthesia [9]. The data obtained using sodium pentobarbital and ether anesthesia agreed with one another for all but that of the basal plasma level of corticosterone. With the sodium pentobarbital, the basal plasma level of corticosterone was determined to be 125 ng/ml. By contrast using the ether anesthesia, the basal level of 25 ng/ml was achieved in agreement with the results of Hilfenhaus [10].

When injected with a saline solution of carrageenan, hypophysectomized rats swell more intensely and for a longer duration than do control ones, cf. **Figure 2**.

Discussion

The carrageenan rat paw edema test was selected as the inflammation model for these experiments because of its popularity in the evaluation of prospective anti-inflammatory compounds [11] and known physiological consequences of carrageenan [9]. Clinically, inflammation is characterized by heat, redness, swelling and pain. In the rat paw edema test, the quantitative measurement of inflammation is assumed to be represented by the amount of edema.

Physiologically, the inflammation process involves a series of events. Increased vascular permeability appears to be one of the more important of these events in that increased permeability permits edema and cellular migration. Vasodilation also occurs, and this results in increased blood flow in the inflammation area. The glucocorticoid steroids counteract the increased vascular permeability in addition to inducing vasoconstriction.

Various types of stresses have been determined to cause the basal plasma level of corticosterone to increase. Therefore, certain experimental precautions were necessary to

avoid the known ones within the experimental animals. The first precaution properly timed the carrageenan injection in order to avoid the afternoon-evening diurnal variation pulse of plasma corticosterone [10]. The second precaution allowed the animal to become comfortable to the conditions of the procedure room prior to experimentation in order to minimize the physical handling stress. The third precaution minimized the time period needed for the blood sampling technique in order to avoid the anesthetic stress-induced pulse of plasma corticosterone [12]. And finally, the fourth precaution avoided anesthetizing the animal during the experiments in order to minimize interference of the anesthetic with corticosterone production.

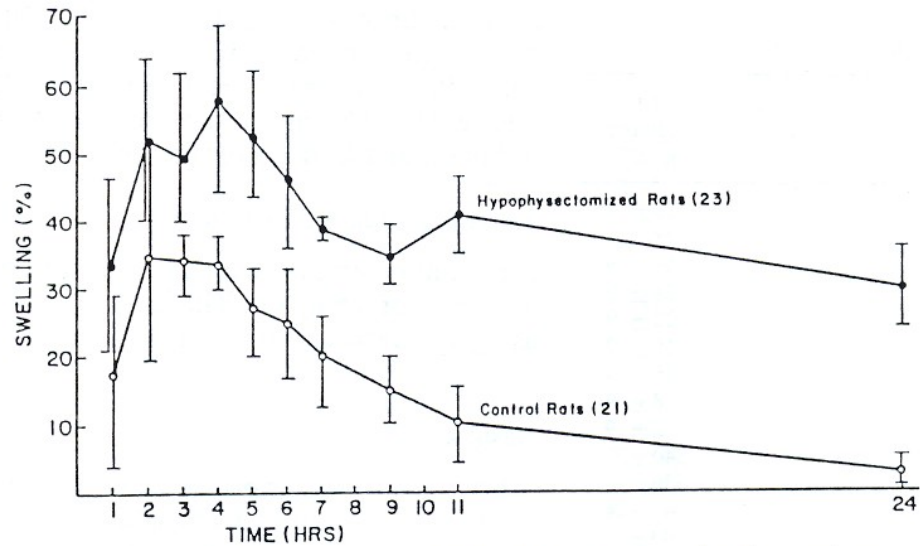


Figure 2
Paw volume responses from a carrageenan injection in hypophysectomized (upper curve) and control rats.

As evident from the data of **Table 1**, both the saline and the carrageenan/saline injections are able to incite the hypothalamus-pituitary-adrenal axis into producing pulsed corticosterone hormone releases. Since the saline injection gave rise to an identical first pulse of corticosterone to that of the saline/carrageenan injection, the first pulse emanates from the injection of the saline solution and not from carrageenan. The second pulse, which begins at 5 hours after injection, is a consequence of carrageenan presence in the paw.

In qualitative agreement with the data of **Table 1**, Leme and Schapoval [2] observed a 67% plasma corticosterone concentration increase in three hours after injection of carrageenan into anesthetized rats. It is unclear whether the diurnal variation or the influence of anesthetized – versus anaesthetized rats – were factored into the experiments.

With the assignment of the second pulse to inflammation caused by carrageenan, the first of the three objectives of this study was achieved. Indeed, inflammation does cause pulsed corticosterone hormone release in a mammal. This is the missing link of the now proposed negative endocrine control system of inflammation in the rat as illustrated in **Figure 3**. The essentials of this Figure agree substantially with that proposed by Leme [3]. Noteworthy, Leme makes an interesting but not definitive argument for the participation of the nervous system. Consequently, use of “endocrine” rather than neuroendocrine” in the title is conservatively deliberate.

The second objective of this study is to evaluate the corticosterone concentration intensity of the inflammation-induced steroid pulse. The comparables are intensities of (1) the basal plasma level and (2) the broad pulse of the diurnal variation. Both of the

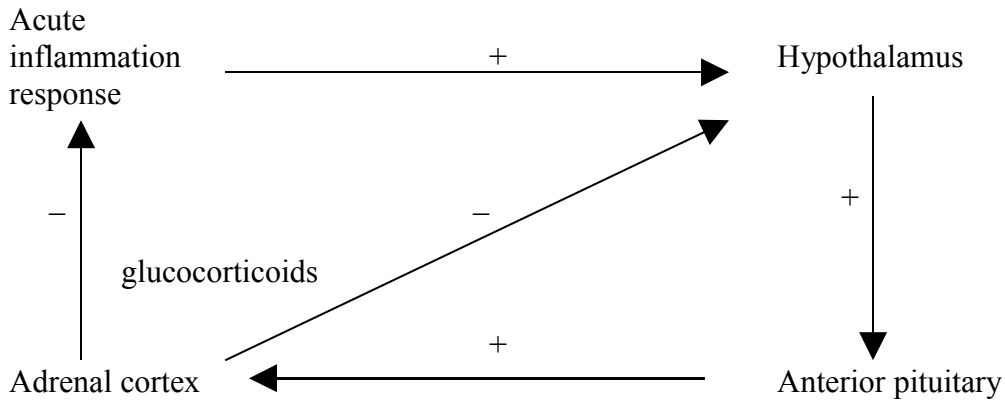


Figure 3 Negative endocrine control system for inflammation in rats. The plus sign implies stimulation and negative sign signifies inhibition.

observed corticosterone pulse intensities resulting from the carrageenan injection are 12-fold that of the basal plasma level of corticosterone and twice that of the maximum intensity of the diurnal variation of the same rat strain [10], cf. **Figure 4**. Therefore, these pulses, particularly that caused by carrageenan, have the potential of causing greater physiological changes than the pulse of the diurnal variation.

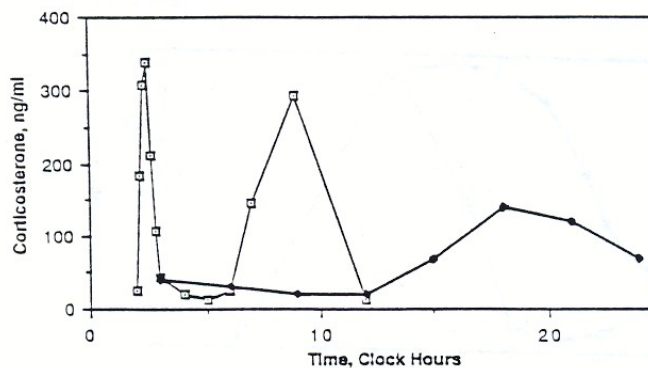


Figure 4 Plasma corticosterone responses from a carrageenan (□) superimposed upon diurnal variation (●) plasma corticosterone concentrations.

The remaining objective of this study is to evaluate the time relationship of carrageenan-induced inflammation with the resultant corticosterone pulse. If the results are superimposed upon the now classic data of Vinegar et al. on the biphasic development of carrageenan edema in rats similarly injected with carrageenan [7], a cause-effect relationship is suggested, cf. **Figure 5**. Since the peak shape of the leading edge of the carrageenan-induced corticosterone pulse approximates that of carrageenan-induced edema response, the edema appears to be responsible for instigating the resultant steroid pulse. In turn, once the latter is initiated its timing is near perfect for terminating the edema response to carrageenan. Likewise, the 20-minute sharp steroid pulse is approximately time to cause the plateau of the biphasic edema curve. Further, the two

observed corticosterone pulses are appropriately timed (20 minutes and 5 hours) to follow the peaks of hyperalgesia at 10 minutes and 3 hours.

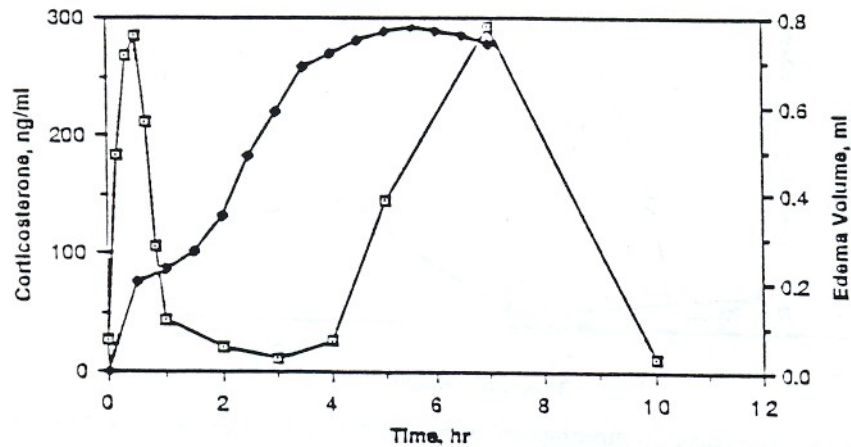


Figure 5
Plasma corticosterone (\square) and edema volume (\blacklozenge) responses from a carrageenan injection.

Since inflammation is presumed to be a desirable response, the body physiology must be such that it will allow inflammation to occur. However, since prolonged inflammation is known to be destructive to the body, the physiology must also have an inflammation termination mechanism. We now wish to propose that the negative endocrine inflammation control system (**Figure 3**) first allows inflammation to occur via the 5-hour delay, i.e., the time between the injection time and the time of initiation of the second corticosterone pulse and initiates the termination of the response 2-3 hours later. The carrageenan rat paw edema is the most popular model for screening potential anti-inflammatory compounds. The data of this study suggest that the edema suppression of the model appears to be the result of the anti-inflammatory action of the compounds tested superimposed upon that of the negative endocrine control system. With the assumption that the negative endocrine control system for inflammation is operational in the rat, it would then be predicted that should the hypothalamus-pituitary-adrenal axis become inoperational, the control system would not function, and inflammation when initiated would not be adequately controlled. In experimental terms, inadequate control should translate into more intense inflammation symptomology resulting from initiation than in the comparable controls.

Experimentally, an inoperable negative endocrine control system for inflammation could be obtained in rats by either adrenalectomy or hypophysectomy. Adrenalectomy causes the experimental animals to become dependent upon steroid supplementation to sustain life. With hypophysectomy, steroid supplementation is unnecessary for sustaining life. Therefore, hypophysectomy was selected for rendering the experimental animal to have an inoperable hypothalamus-pituitary-adrenal axis. When hypophysectomized Sprague-Dawley rats are injected with carrageenan in the usual manner, the resulting edema is more intense and last longer than it does in control rats, cf. **Figure 2**. The data is consistent with an operational negative endocrine control system in the rat.

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