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Establishing a Protocol for Dexamethasone Suppression Testing in Mice

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**ESTABLISHING A PROTOCOL
FOR DEXAMETHASONE SUPPRESSION TESTING
IN MICE**

submitted by

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Senior Honors Thesis

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Abstract

Dexamethasone is a synthetic glucocorticoid which can be used to suppress the levels of glucocorticoids in the body. Thus the dexamethasone suppression test is used to evaluate the function of the negative feedback control of the hypothalamic-pituitary-adrenal axis (HPA axis). While a protocol for dexamethasone suppression testing in rats and humans has been established, an acceptable program for administration of dexamethasone to mice was lacking. In this study, mice transgenic for growth hormone, which are hypothesized to exhibit abnormal HPA control, and their normal littermates were used in three different trials in an attempt to examine the effects of dexamethasone on adrenal corticosterone secretion in mice. The first trial involved PEPCK-hGH transgenic mice and their normal siblings. First, an orbital sinus puncture under ether anesthesia was taken at 0700 hrs in order to obtain a basal level of corticosterone. A subcutaneous injection of 0.2 ug dexamethasone 21-phosphate (DEX)/100g body weight was administered at 0800 hrs, and another orbital sinus puncture was taken 4 hours later. The second trial followed similar procedure but used MT-hGH transgenic mice and their normal siblings. It was concluded that the dosage of dexamethasone was insufficient in these trials since levels of corticosterone were higher at four hours post injection than at the basal time. Therefore a tenfold increased dosage (2.0 ug DEX/100g body weight) was used for the third trial. Normal siblings of transgenic mice were randomly assigned to either a dexamethasone or saline injection group. In addition to basal levels, corticosterone levels were measured at two and four hours post injection. The results of this experiment showed that plasma corticosterone levels in mice treated with dexamethasone were lower than in the saline control group. However, levels at post times for both groups were still elevated in comparison to basal levels. At four hours post, the dexamethasone group showed levels near

the basal while the saline group was still significantly elevated. Therefore, administering 2.0ug dex/100g body weight significantly suppresses levels of corticosterone in mice. The issue still remains, however, why levels increased at two and four hours post injection. Further study is necessary to determine if this rise is a response due to the stress of ether, orbital sinus puncture, and handling or if it is perhaps a natural rise preceding the peak of corticosterone levels in the early evening.

Introduction

The hypothalamic-pituitary-adrenal axis (HPA axis) plays a vital role in coordinating daily activities and regulating an animal's response to stressors [2]. This study focuses primarily on HPA axis mediation of glucocorticoid levels. In response to a perceived stress, the release of corticotropin releasing factor (CRF) is triggered in the mediobasal hypothalamus. CRF then travels thru a portal system to the anterior pituitary where it supports the synthesis and release of adrenocorticotrophic hormone (ACTH). In turn, ACTH stimulates the synthesis and release of glucocorticoids from the adrenals. Once in the circulation, glucocorticoids, such as corticosterone in mice or cortisol in humans, lead to lipolysis, glycogenolysis, and protein catabolism. Hence, the animal has increased energy available to cope with a stressor. [4] Additionally, the glucocorticoids act on receptors in the hypothalamus and pituitary to inhibit the release of CRF and ACTH [6]. Thus, the HPA axis operates as a closed-loop negative feedback system in order to regulate glucocorticoids as shown in Figure 1.

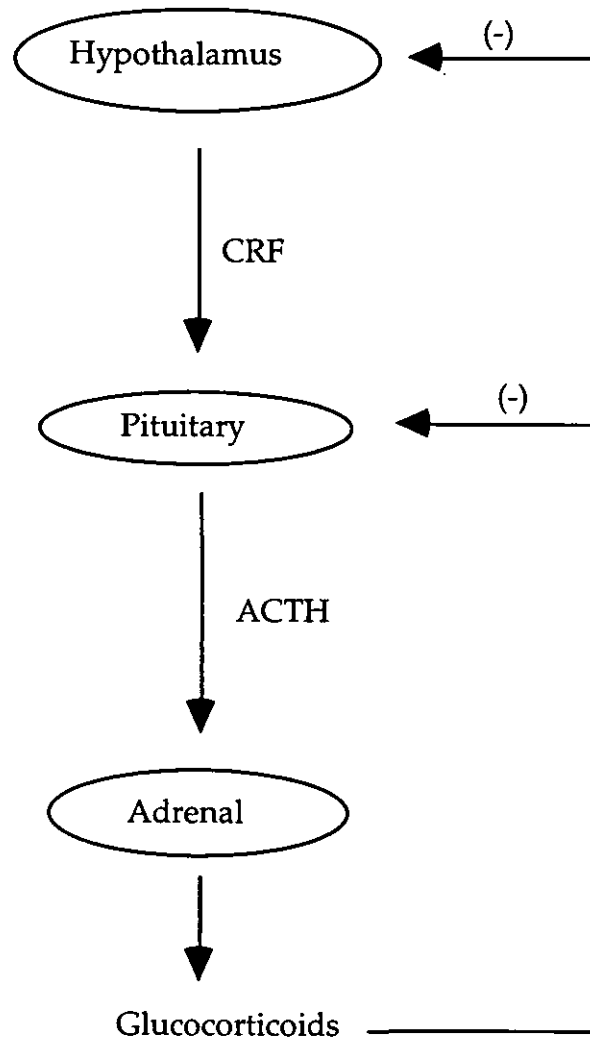


Figure 1: The hypothalamic-pituitary-adrenal feedback system

Studies have shown that there are various conditions that seem to alter the functioning of the HPA axis by disrupting the feedback system and consequently leading to chronically elevated glucocorticoid levels. In humans these conditions include depression, Alzheimer's disease, and other dementias [4,6]. The effects of aging on the HPA axis has received much attention and numerous

studies have shown elevated basal glucocorticoid levels as well as increased HPA axis reactivity to stress in aged rats [2,3,4,5,8]. Prolonged elevation of glucocorticoids are harmful due to its catabolic effects. Suppression of anabolic processes, muscle atrophy, decreased sensitivity to insulin, increased risk of steroid induced diabetes, hypertension, hyperlipidemia, hypercholesterolemia, arterial disease, amenorrhea, infertility, impairment of growth and repair of tissue, prolonged immune suppression, and osteoporosis are all associated with chronically high levels of glucocorticoids [4].

In addition to age and dementia effecting HPA axis activity, it has been shown that several strains of mice transgenic for growth hormone also demonstrate HPA functional abnormalities. Bartke et al. found corticosterone levels in these transgenic mice that were congruent to levels found in normal mice of a much greater chronological age [1]. Furthermore, these transgenic mice show a prolonged elevation of glucocorticoid levels in response to stress [Steger, R.W. and Bartke, A. , unpublished]. Therefore, it seems mice transgenic for growth hormone exhibit the effects of aging on the HPA axis prematurely.

In order to explain the increased HPA axis activity in mice transgenic for growth hormone it has been hypothesized that the glucocorticoid feedback mechanism does not function properly in these animals. Dexamethasone (DEX) is a synthetic glucocorticoid that suppresses CRF, ACTH, and thus plasma corticosterone levels in the same manner as corticosterone. Therefore, dexamethasone suppression tests can be used to assess the functioning of the glucocorticoid feedback system, which is necessary to test this hypothesis.

Dexamethasone suppression tests are commonly used in humans for clinical psychiatry and a protocol for such testing in rats has been established [6,9]. However, an acceptable program for administering dexamethasone to mice was lacking. Therefore, as a first step in studying the HPA axis feedback control

in mice transgenic for growth hormone, the goal of this project was to establish a protocol for dexamethasone suppression testing in mice.

Materials and Methods

Transgenic mice were originally produced by microinjection of hGH gene fused to rat phosphoenolpyruvate carboxykinase promoter/regulator (for PEPCK-hGH mice) or mouse metallothionein-I promoter (for MT-hGH mice) into the pronuclei of fertilized mouse eggs as previously described [9]. Lines of transgenic mice were then produced by mating a male mouse positive for hGH expression with B6C3 F₁ hybrid females as previously described [9].

Mice used in this study were housed 4 to 5 per cage in a temperature controlled room (22° C) on a 12:12 light:dark schedule (lights on at 0600h). Food (TekLad 6% Rat/Mouse Diet 002, containing a minimum of 24% protein and 6% fat and a maximum of 5% fiber, Teklad Premier; Harlan Sprague-Dawley, Madison, WI) and tap water were provided ad libitum. In each trial, the mice were handled four of seven days during the week prior to experimentation.

TRIAL I

Mice used in this trial were PEPCK-hGH transgenic males or their normal male siblings. At 0600h mice were transferred from the vivarium to the laboratory. In order to prevent the stress of transfer from confounding results, the mice were not handled in the new environment for one hour. Then, beginning at 0700 hours each mouse was anesthetized with ether and basal blood samples were rapidly taken by orbital sinus puncture. EDTA (10.0 uL) was used in each sample as an anticoagulant. Subcutaneous injections of dexamethasone 21-phosphate (Sigma Pharmaceuticals, St. Louis, MO.) dissolved in saline began at 0800h. Each mouse received 0.2 ug DEX/100 g.b.w. according to the procedure for rats previously described [7]. Four hours post injection with DEX

animals were anesthetized and blood samples taken for the second time. All blood samples were centrifuged at 2500 g for 15 minutes and the separated plasma was frozen until analysis. Animals were allowed to recover and then returned to the vivarium. The mice continued to be handled throughout the following week.

Seven days after the original experiment, mice were randomly assigned to control or dexamethasone groups with an approximately equal number of normals and transgenics in each group. The mice were transferred at 0700h this time, with injections beginning at 0800h. The injections followed the same procedure as above except that controls received saline only. Four hours post injection the animals were sacrificed by decapitation and trunk blood collected. Again, EDTA (100 uL for normal mice or 200uL for transgenic mice) was used as an anticoagulant, samples were centrifuged, and plasma frozen until analysis.

TRIAL II

Trial II followed the identical procedure as described for Trial I except that the mice used were males transgenic for MT-hGH or their normal siblings.

TRIAL III

For Trial III the normal male siblings of mice transgenic for growth hormone were used. The mice were randomly assigned to either control or dexamethasone groups. Then the members of each of those groups were randomly assigned to either a 2 or 4 hour group (indicating the time elapsed between injection and blood sample collection) such that the 2 and 4 hour groups consisted of approximately the same number of controls and dexamethasone group members. Mice were transferred at 0600h. Animals were anesthetized with ether and blood samples collected by orbital sinus puncture at 0700h. Injections began at 0800h. This time the dosage was 2.0 ug DEX/100g.b.w. for the dexamethasone group while controls received saline injections. Then the 2

hour group animals were sacrificed by decapitation and trunk blood collected beginning at 1000h. The 4 hour group members were similarly sacrificed beginning at 1200h. Samples were handled as described above.

CORTICOSTERONE ASSAY

Serum samples were analyzed for corticosterone concentration by radioimmunoassay using a commercially available kit (ICN Pharmaceuticals, Inc., Cost Mesa, CA.). Instructions provided with the kit were followed with the following modifications:

1. The 1:200 dilution of mouse serum with steroid diluent was made by taking 5.0 uL of sample to 1.0 mL.
2. All of the remaining volumes used throughout the procedure were divided by two.

The assay is effective for concentrations ranging from 25 ng/ml to 1000 ng/ml with an intraassay variation of approximately 5%. All samples from a single trial were run in duplicate in the same assay.

Additionally, an assay was run in order to determine the amount of assay cross-reaction with dexamethasone to ensure that the assay was appropriate for dexamethasone suppression testing. This was done by using concentrations of dexamethasone ranging from 0.10 ng to 100 ng DEX in a corticosterone assay.

Results

The antibody used in the corticosterone assay showed no cross reaction with dexamethasone.

TRIAL I

Plasma corticosterone levels were elevated in PEPCK-hGH transgenic mice as compared to normals (Fig 2). Plasma corticosterone did not decrease, and actually showed an increase, after injection of 0.2 ug DEX/100 g.b.w. for both

groups of mice. Animals injected with 0.2 ug DEX/100 g.b.w. exhibited slightly higher levels of plasma corticosterone than those treated with saline but the results were not significant.

TRIAL II

Similar to results in Trial I, corticosterone levels in normal and MT-hGH transgenic mice were increased after injection with 0.2 ug DEX/100 g.b.w. (Fig 4). Animals injected with 0.2 ug DEX/100 g.b.w. had slightly lower levels of corticosterone relative to animals injected with saline (Fig 5).

TRIAL III

As depicted in Figure 6, mice treated with 2.0 ug DEX/100 g.b.w. exhibited significantly lower plasma corticosterone levels at two and four hours post injection than did mice treated with saline. Also, animals from both the saline and DEX groups showed elevated corticosterone levels at times after injection in comparison to basal levels, but this elevation was only significant for the saline treated mice.

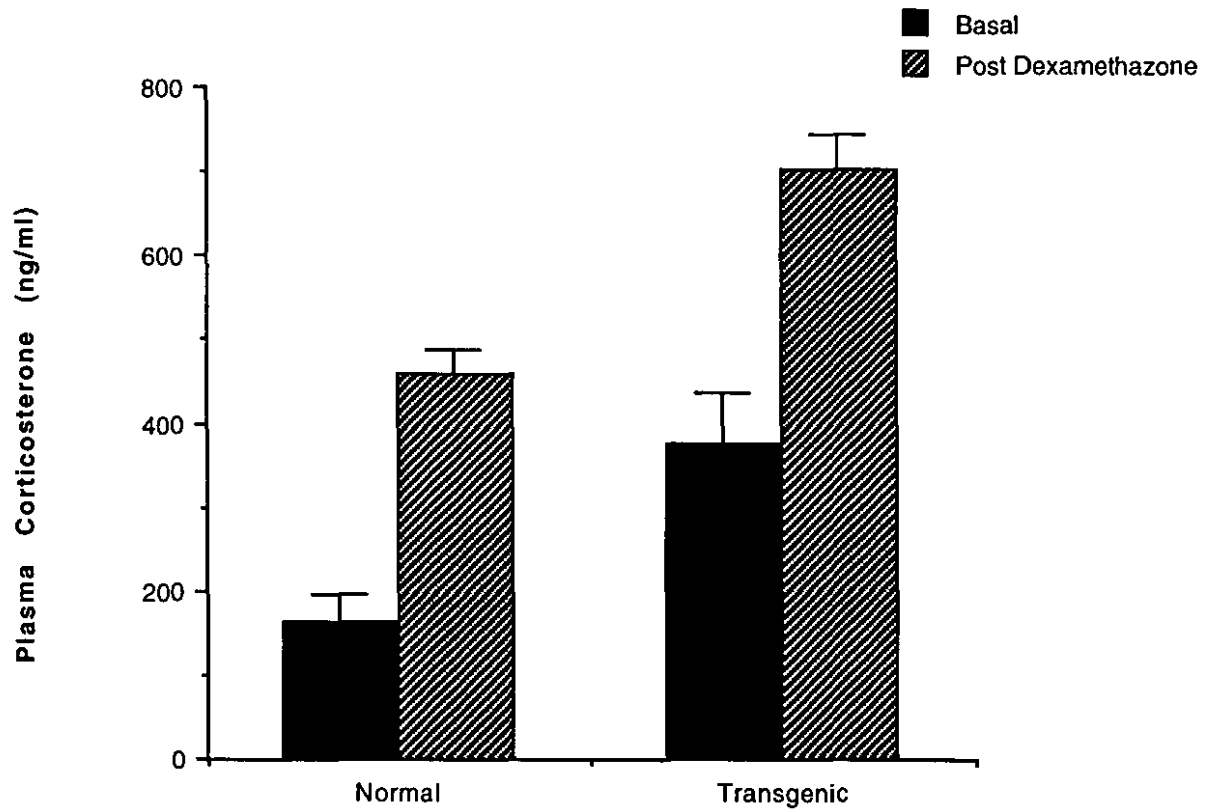


Figure 2: Plasma corticosterone levels of normal and PEPCK-hGH transgenic mice at basal time and 4 hours after injection of 0.2 ug DEX/100 g.b.w. (mean±SEM)

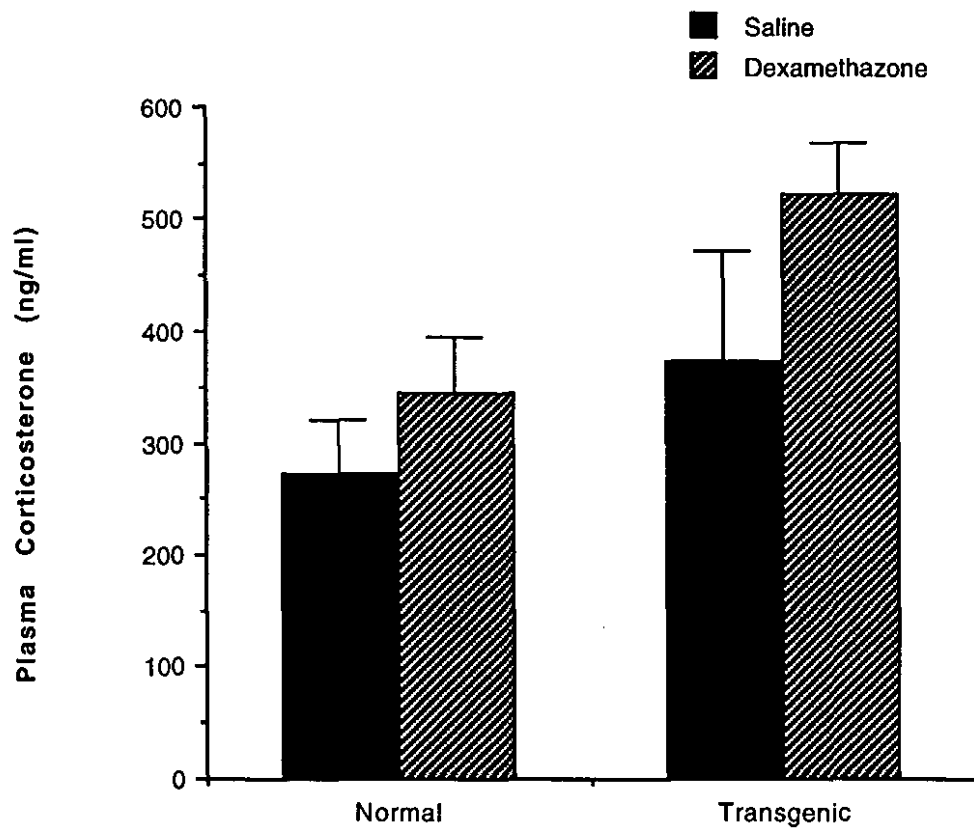


Figure 3: Plasma corticosterone levels of normal and PEPCK-hGH transgenic mice 4 hours after injection with saline or 0.2 ug DEX/100 g.b.w. (mean \pm SEM)

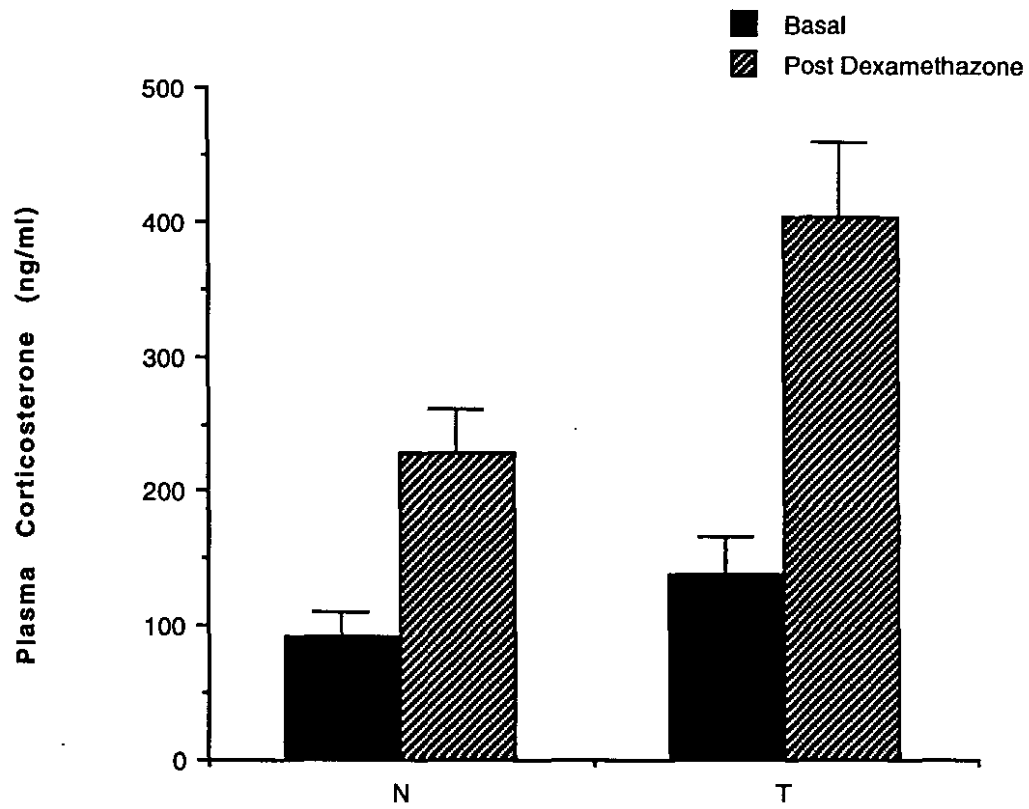


Figure 4: Plasma corticosterone levels of normal and MT-hGH transgenic mice at basal time and 4 hours after injection with 0.2 ug DEX/100 g.b.w. (mean \pm SEM)

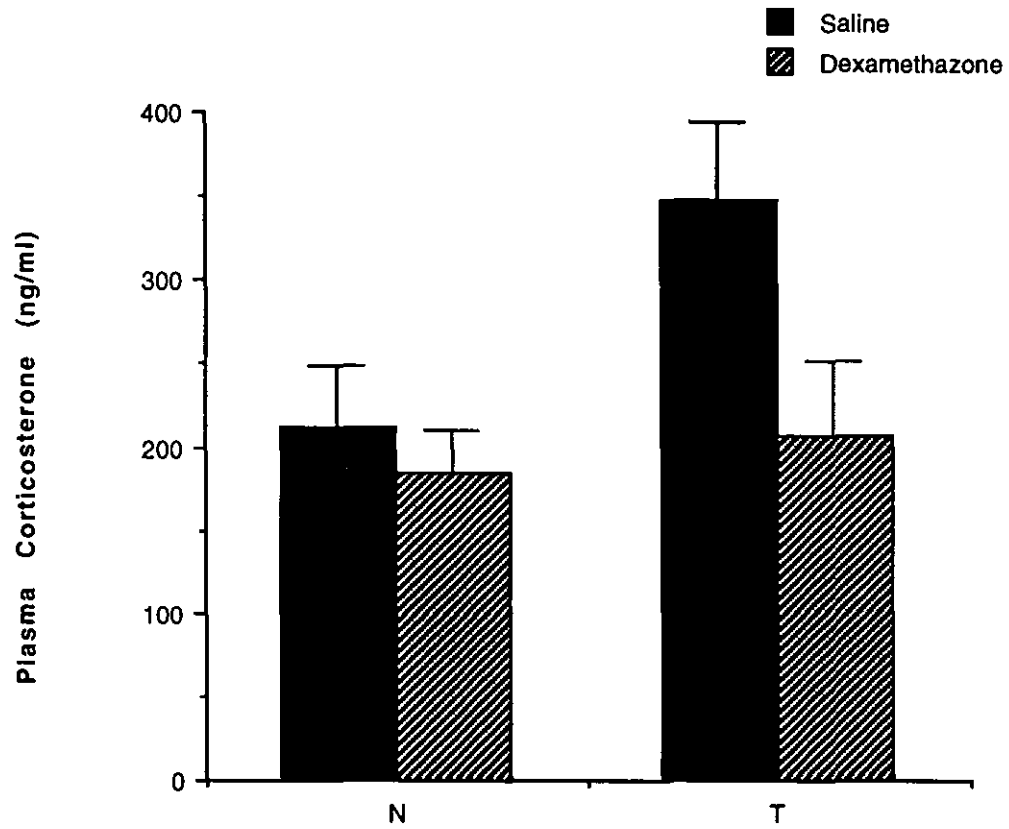


Figure 5: Plasma corticosterone levels of normal and MT-hGH transgenic mice 4 hours after injection with saline or 0.2 ug DEX/100 g.b.w. (mean \pm SEM)

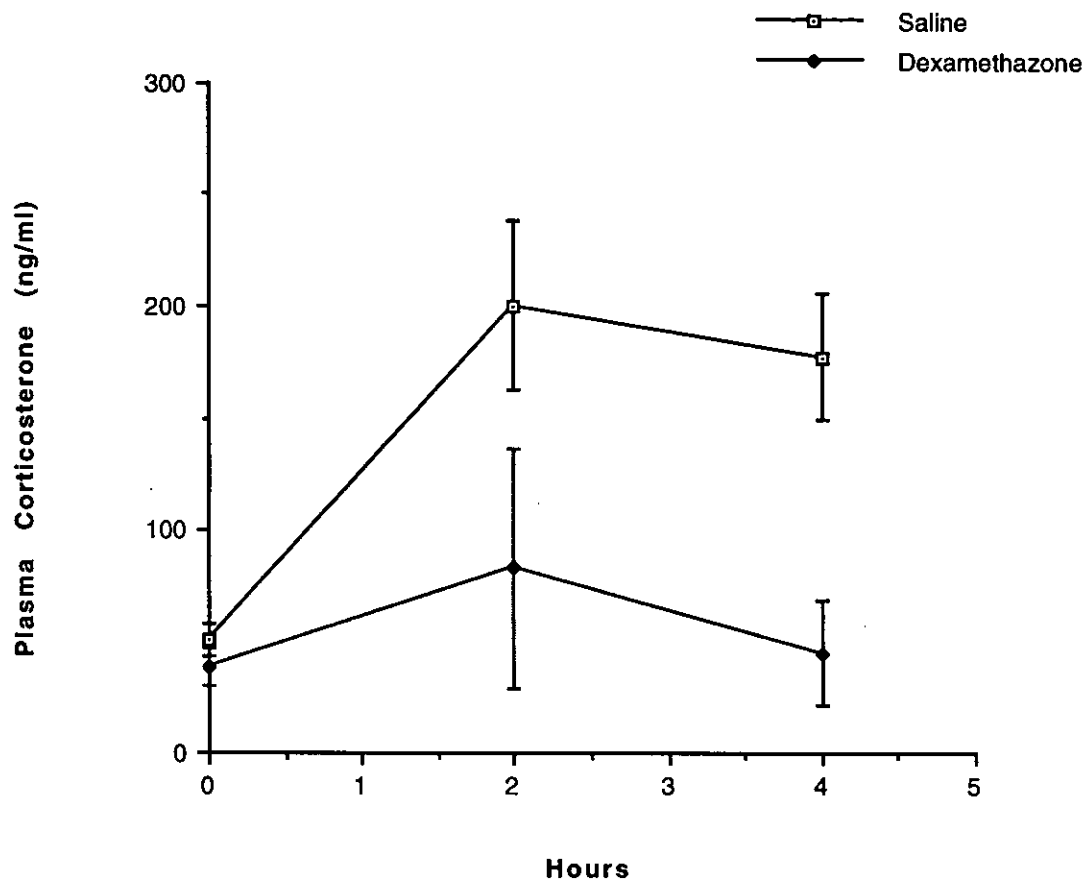


Figure 6: Plasma corticosterone levels of normal mice prior to and two or four hours after injection with saline or 2.0 ug DEX/100 g.b.w. (mean±SEM)

Discussion

In an attempt to study the functional abnormalities of HPA axis activity found in mice transgenic for growth hormone, several trials of dexamethasone suppression tests were conducted. The lack of suppression of plasma corticosterone levels seen in Trial I and Trial II indicates that the 0.2 ug DEX/100 g.b.w. dosage recommended for rats by Oxenkrug is insufficient for suppressing corticosterone levels in mice. This may be due to the increased metabolic rate of mice. However, the significant amount of glucocorticoid suppression exhibited

by the mice treated with DEX in Trial III indicates that a 2.0 ug DEX/100 g.b.w. dose is sufficient for corticosterone suppression testing in mice.

The results of this experiment raise many questions and provide direction for further research. The efficiency of the negative feedback control of adrenal corticosterone secretion in mice transgenic for growth hormone has yet to be assessed by dexamethasone suppression testing; but the procedure described in Trial III can be used for such assessment. Also, data from Trial I and Trial II provide further evidence of elevated plasma corticosterone levels in these transgenic mice relative to normal mice.

Of further interest is the rise in plasma corticosterone levels over the four hour testing period illustrated in Figure 6. Most likely these animals were stressed by ether, orbital sinus puncture, or injection causing increased corticosterone secretion. Rapid sampling of blood after anesthetization should not have allowed sufficient time for circulating levels of corticosterone to be affected, but after the first sampling the animals may have had memory of the stress and reacted to handling. Further study is necessary to determine if this rise in plasma levels of corticosterone is indeed due to stress or some other factor such as a natural daily rise preceding the peak of corticosterone levels in the early evening. Perhaps if blood samples and injections could be done via cannules this confounding of results might be avoided. However, cannulation is very tedious and impractical in mice.

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