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Inhibition of Corticosteroid-Binding Globulin Caused by a Severe Stressor Is Apparently Mediated by the Adrenal but not by Glucocorticoid Receptors

Octavi Martí, Miquel Martín, Amadeu Gavaldà, Merce Giralt, Juan Hidalgo, Brend R.-S. Hsu, Robert W. Kuhn, and Antonio Armario

Departament de Biología Celular y de Fisiología, Facultad de Ciencias, Universidad Autónoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

The effect of stress on serum corticosteroid-binding globulin (CBG) was studied in adult male Sprague-Dawley rats. CBG was measured either by a homologous radioimmunoassay (RIA) or by a binding assay (BA) using 3H-corticosterone. Exposure of adult male rats to a severe stressor such as immobilization (IMO) for 1 h did not alter serum CBG levels, but a significant decrease was found after 6 and especially 24 h IMO. This decrease was not observed after 24 h exposure to a milder treatment such as food and water deprivation. The effect of different periods of exposure to two stressors, IMO or restraint, was also studied. The following results were obtained: serum CBG levels were reduced by IMO, but not by restraint; IMO-induced reduction of CBG levels was always observed 24 h after starting exposure to IMO, independently of the actual period of exposure to the stressor; and IMO-induced inhibition of CBG was proportional to the hours of exposure to the stressor. Although IMO-induced inhibition of CBG was prevented by adrenalectomy, a role for glucocorticoid acting through their classical type II receptors is unclear as far as treatment of rats with the glucocorticoid receptor antagonist RU486 (100 mg/kg) did not prevent the inhibition caused by IMO. The present data clearly indicate that acute exposure to a stressor is able to decrease CBG levels provided that duration of exposure to the stressor and its intensity are high and that the effect is tested at least 6 h after the onset of stress. The effect appears to be mediated by some adrenal factor(s) other than glucocorticoids.

Key Words: Corticosteroid-binding globulin; immobilization; restraint; stress; pituitary–adrenal hormones.

Introduction

Selye activation of the pituitary–adrenal (PA) axis is considered as one of the most relevant properties of stressful stimuli. For this reason, the influence of acute and chronic stress on the PA axis has been extensively investigated. Exposure of animals to acute stressors induces initially a potent response followed by a progressive return to prestress levels in spite of continuous exposure to the stressor *(1,2)*. Similarly, daily exposure of rats to the same stressor quite often results in habituation/adaptation characterized by a lower response to the stimulus *(3,4)*.

This reduction of the PA response to both acute long-term stressors and chronic intermittent stressors is obviously the result of a complex pattern of changes at different levels of the axis *(2,4)*. In this regard, the possible contribution to adaptation of the changes in the corticosteroid binding capacity of blood has not been well characterized. The role of corticosteroid-binding globulin (CBG), the protein that specifically binds circulating corticosteroids, on biological activity of corticosteroids has gained renewed interest in view of the fact that CBG appears to exist in different tissues, to be involved in the control of corticosteroid uptake by cells and to act as a biological significant signal *(5,6)*, in addition to, or in opposition to its classical role in controlling the amount of free corticosteroids in blood.

Changes in circulating CBG are expected to occur after stress because glucocorticoids appear to play an inhibitory role in the control of hepatic CBG synthesis *(7–13)*, and stress are natural situations in which glucocorticoid release always occur. However, the influence of stress on corticosteroid-binding activity in plasma is at present unclear. Savu et al. *(14)* reported that pharmacological induction of inflammation, which is accompanied by glucocorticoid release, reduced CBG activity in rats. Conversely, Bassett *(15)* found short-term increases in the corticosteroid binding activity of plasma after short-term acute stress. In addition, a reduction of plasma CBG levels after acute tailshock has very recently been reported that is apparently not mediated by corticosterone *(16)*. In view of these conflicting data about the effects of stress on CBG levels and the

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Author to whom all correspondence and reprint requests should be addressed: Dr. Octavi Martí, Departament de Biologia Celular y de Fisiología, Facultad de Ciencias, Universidad Autónoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

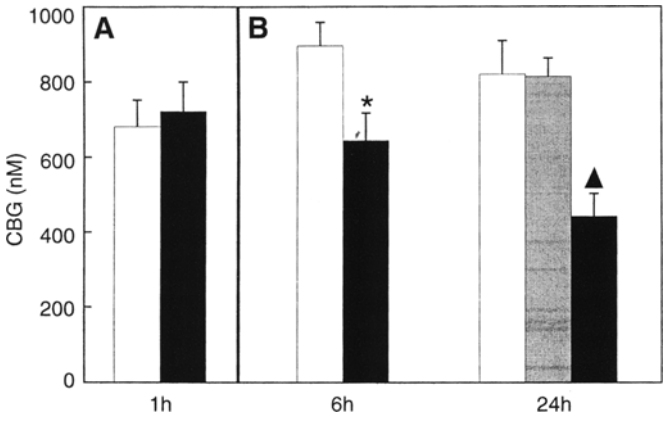

Fig. 1. Effect of immobilization stress on serum CBG levels (Exp. 1A and 1B). Means and SEM (n = 5–6 per group) are represented. Open bars indicate controls, shaded bars food and water deprived (FWD) animals and closed bars immobilized animals. The numbers under bars indicate the hours of exposure to the stressor; (*) $p < 0.05$ vs 6 h control group (Student's t-test), ▲ $p < 0.05$ vs 24 h control or FWD groups (SNK test). The rats were killed between 10.00 and 11.00 h (1 h and 24 h groups) or between 15.30 and 16.00 h (6 h groups).

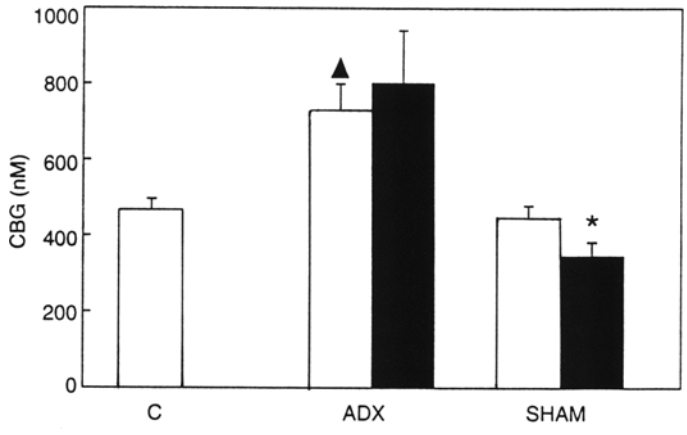

Fig. 2. Effect of adrenalectomy on CBG response to 18 h IMO. Means and SEM (n = 57 per group) are represented. C = intact (no surgery) rats; SHAM = sham-adrenalectomized rats; ADX= adrenalectomized rats. Open bars indicate prestress and closed bars stress levels and thus, the same rats serves as their own controls (C group was not subjected to IMO after the first blood sampling, *see* Results). Samples were always obtained between 10.00 and 11.00 h; (▲) denotes significant difference with respect to C and SHAM rats before stress (SNK test, $p < 0.05$); (*) denotes significant difference with respect to appropriate prestress values (paired Student's t-test, $p < 0.05$).

possible role of glucocorticoids, a series of experiments were designed to investigate the influence of different lengths and intensities of stressful situations on circulating levels of CBG.

Results

Exposure to 1 h IMO did not alter CBG levels (Fig. 1A). However, a significant decline was observed after 6 h of stress in Fig. 1B ($p < 0.05$ vs corresponding control group). When the effect of 24 h IMO was studied, an additional food and water deprived group was included since the immobilized animals had no access to food and water. One-way analysis of variance (ANOVA) ($p < 0.004$) followed by posthoc comparisons [Student–Newman–Keuls—test (SNK test)] revealed that 24 h of IMO, but not 24 h of food and water deprivation, significantly decreased CBG levels.

The effect of adrenalectomy on CBG response to 18 h IMO was studied in Exp. 2. A blood sample of nonstressed intact was first obtained, SHAM-ADX and ADX rats. After that, SHAM and ADX rats were subjected to 18 h IMO and sampled again. Intact rats were not subjected to 18 h IMO, but sampled again at the same time as SHAM and ADX rats to control for a possible effect of the previous sampling *per se*. The one-way ANOVA of CBG values before stress revealed a significant effect of adrenalectomy ($p < 0.001$) in that CBG levels were higher in ADX than in intact and SHAM rats (SNK test). In intact rats, no effect of sampling was found (first sampling= 469 ± 28; second sampling= 558 ± 47 n*M*). After exposure to 18 h IMO, a significant decline in serum CBG was observed in SHAM (paired t-test, $p < 0.05$), but not in ADX rats, when compared with appropriate prestress values (Fig. 2).

Table 1
Effect of Glucocorticoid Receptor Antagonist RU486 on IMO-Induced Changes in Serum CBG Levels (n*M*)

Treatment	Control	18 h IMO
Vehicle	791 ± 38 (11)	528 ± 75 (8)
RU486	674 ± 65 (6)	481 ± 49 (8)

Means ± SEM are represented. The number of animals per group are in parentheses. The two-way ANOVA revealed that IMO, but not RU486 significantly decreased serum CBG ($p < 0.001$).

In Exp. 3, rats were given vehicle or the glucocorticoid receptor antagonist RU486 and 2 h later they were left undisturbed or subjected to 18 h IMO. The two-way ANOVA revealed a significant effect of IMO ($p < 0.001$), which decreased CBG levels, but no effect of RU486 administration (Table 1).

The aforementioned data suggested that a prolonged period of exposure to a stressor was needed to inhibit CBG, and that not all stressors were able to decrease CBG. The need for a prolonged period of exposure to stressors has been questioned by recent findings that demonstrate that a lag period of several hours is needed in order for stress exposure (e.g., 1 h shock) to reduce plasma CBG levels *(16)*. The aim of Exp. 4 was twofold: to compare the effects of two stressors (IMO in wooden boards vs restraint in tubes) differing in the intensity of PA activation they elicit *(17)*; and to compare different periods of acute exposure to IMO and different periods of resting on CBG and corticosterone levels. The

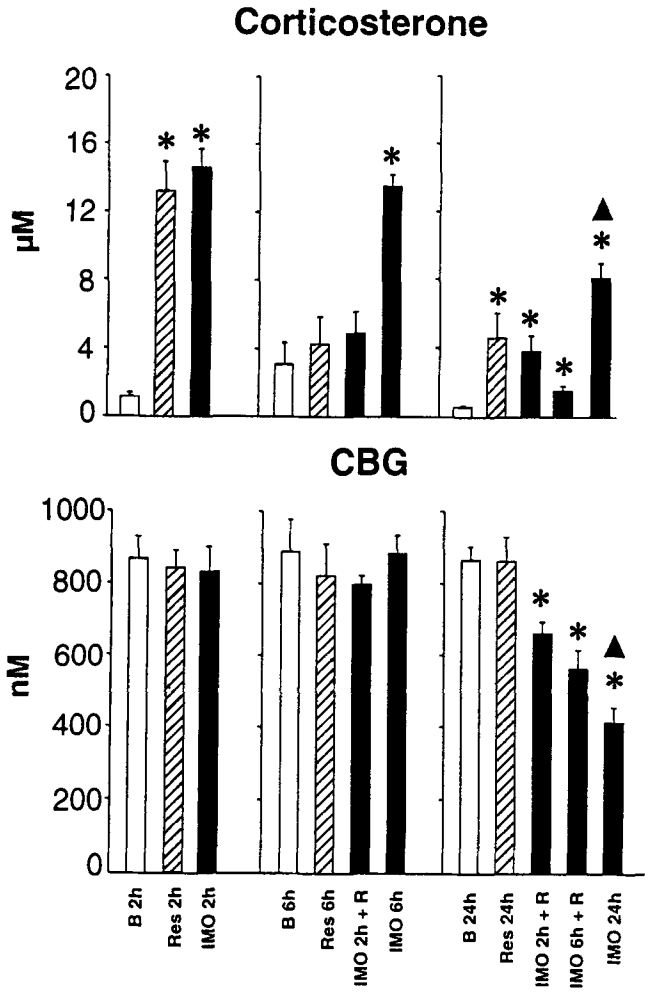

Fig. 3. Effects of different periods of exposure to IMO or restraint on serum corticosterone and CBG levels ($n = 6$–8 per group). Open bars indicate controls (B), shaded bars restrained rats (Res), and closed bars IMO rats. R identifies those groups of rats subjected to IMO for the number of hours indicated and killed along appropriate control and continuously stressed rats. The three killing periods are separated by vertical bars and were at 11.00, 16.00, and 10.00 h, respectively. Within the same killing period, (*) denotes significant difference with respect to controls, (▲) significant difference with respect to the other groups (corticosterone) or with respect to 2 h IMO plus resting (CBG) (SNK test, $p < 0.05$).

one-way ANOVA for each of the three (2 h, 6 h, and 24 h) periods studied revealed in the three cases a highly significant effect of the treatments on serum corticosterone ($p < 0.001$). Posthoc comparisons (SNK test, Fig. 3) revealed that:

1. Two hours of exposure to IMO or restraint significantly increased serum corticosterone as measured immediately after stress;
2. In the 6-h period, only rats continuously immobilized for 6 h showed serum corticosterone levels higher than controls;
3. In the 24-h period, again the effect of continuous exposure to IMO was greater than that of any other stress procedure (including restraint);
4. In the 24-h period, all IMO groups (regardless of the hours of resting after IMO) showed higher serum corticosterone levels than control rats.

When the same statistical analysis was applied to CBG (Fig. 3), the one-way ANOVAs revealed a significant effect of the treatments in the 24 h groups only ($p < 0.001$). This means that 2 or 6 h after initial exposure to IMO no changes in CBG were observed regardless of whether or not IMO was discontinued. In contrast, 24 h after initial exposure to IMO, all the rats showed reduced CBG levels as compared to controls, the degree of reduction being positively related to the hours of exposure to IMO. Restraint did not modify CBG in any case.

Discussion

The present results and other unpublished data from the laboratory clearly indicate that short-term exposure (e.g., 1 h) to a severe stressor, such as IMO in woodboards, did not change serum CBG concentration as measured by RIA. However, after 6 h of exposure to IMO a significant decrease was found, the effect being more marked after 24 h stress. In another experiment (not reported), 24 h restraint did not alter CBG levels as measured by the same method. The reduction of CBG levels was not observed by exposing the rats to 24 h of food and water deprivation and, therefore, inhibition of CBG was not because of food and water deprivation accompanying this stressor, but to IMO *per se*. Although IMO rats were also deprived of sleep, it is unlikely that this might be the factor responsible for the inhibition of CBG as: No effect of continuous restraint in tubes for 24 h has been observed despite possible perturbation of sleep; rats subjected to IMO for 2 or 6 h and then returned to their home cages (*see* Exp. 4) had the opportunity to sleep, but a significant decrease in serum CBG levels was again observed in those rats on the day after exposure to IMO. Since restraint in tubes elicited a lower PA activation than IMO *(17)*, the present data thus suggest that the intensity of the stressors might be important to inhibit CBG.

It has very recently been demonstrated that a lag period of several hours is sufficient in order for 2 h shock to reduce CBG levels in plasma *(16)*. In view of these results, a new experiment was designed in order to compare restraint and IMO, and also different schedules of exposure to IMO. In this experiment, a binding assay was used as RIA was no longer available. It was confirmed that IMO is a stronger stressor than restraint as evaluated by the serum corticosterone levels measured over the 24-h period. In addition, the results by Fleshner et al. *(16)* were also confirmed in that 2 h of exposure to IMO was enough to reduce CBG levels when measured 22 h later. However, by increasing the hours of exposure to IMO, a greater inhibition of CBG was observed (always 24 h after initial exposure to the stressor), which suggests that CBG reduction is not an all or none phenomenon, but is sensitive to the length of exposure to a severe stressor. Exposure to various restraint protocols did not modify CBG, in accordance with the authors' unpublished data measuring CBG by RIA. Therefore, it

appears that RIA and binding data are quite consistent, the only exception being the lack of effect of 6 h IMO on CBG. Apparently, the period of time needed to obtain reproducible results might be longer than 6 h. The results from Exp. 4 also suggest that the lack of effect of restraint on CBG levels is not merely a consequence of integrated corticosterone levels over the 24-h period since inspection of the data clearly reveals that corticosterone release was not smaller in continuously restrained rats than in the discontinuously IMO rats.

The present results are also consistent with those reported by Savu et al. *(14)* who found that administration to rats of the inflammatory agent turpentine reduced the corticosteroid binding capacity of the serum fraction corresponding to CBG. In contrast, Bassett *(15)* has reported that exposure of rats to footshock increased the specific binding of corticosteroid in serum at 15, 35, and 45 min after stress, but not at 25 min. These changes were absent in ADX rats, and the initial, but not the second peak was also found after adrenocorticotrophic hormone (ACTH) administration. Since the rats were subjected to 1 h IMO, a period longer than that used by Bassett *(15)*, it seems possible that some CBG changes took place in the rats of the current experiments before the first hour. However, complex pattern of changes in CBG is difficult to explain. The use of binding instead of direct measurement of the protein is not the reason for the discrepancies as quite similar results have been obtained with binding and RIA assays and Fleshner et al. *(16)* also used a binding procedure. Although it is not likely, other factors might have contributed to the discrepancies. First, corticosteroid-binding proteins different from CBG have been described *(18,19)*, and stress might induce the release of these proteins. Second, some factors altered by stress might affect the binding properties of CBG. In this regard, it has been found that free fatty acids, which are released during stress, alter the binding properties of human CBG *(20,21)*.

The involvement of glucocorticoids on stress-induced inhibition of CBG appeared possible as adrenalectomy has been reported to increase and glucocorticoids to decrease plasma CBG levels *(7,8,10–13)*. Also in humans, hypercortisolism is accompanied by reduced CBG levels *(22)*. The authors, therefore, decided to study the possible role of glucocorticoids on IMO-induced inhibition of CBG by means of two different, but complementary approaches: adrenalectomy and administration of the glucocorticoid receptor antagonist RU486. It was found that adrenalectomy increased, as expected, serum CBG levels and also prevented the inhibition caused by prolonged exposure to IMO in SHAM-ADX rats. However, blockade of glucocorticoid receptors with a high dose of the antagonist RU486 neither altered CBG levels in nonstressed rats nor prevented the inhibition caused by IMO. Since it was demonstrated that even a 10 mg/kg dose of RU increased ACTH levels in rats *(23)*, and a dose of 100 mg/kg caused a great increase of stress levels of ACTH in mice (unpublished data), the dose of RU used likely blocked glucocorticoid receptors in the present work.

Therefore, glucocorticoids might alter CBG through a mechanism independent of classical glucocorticoid type II receptors or, alternatively, some adrenal factor other than glucocorticoids might be responsible for the inhibition of CBG during stress. The second hypothesis agrees with the lack of effect of acute corticosterone administration on CBG reported by Fleshner et al. *(16)*. Taking into account that RU486 is also an antagonist of progesterone receptors and stress increases circulating progesterone *(24,25)*, the current data argue against a role of progesterone on stress-induced inhibition of CBG. The lack of effect of RU486 is not necessarily in contrast with the findings that ADX increases and exogenous glucocorticoid administration decreases CBG in both ADX and normal animals, particularly taken into account that a minimum period of 18–24 h of glucocorticoid release is apparently needed to cause inhibition of CBG (*see* for instance refs. *26* and *27*) and that most published data have been obtained after various days of adrenalectomy or glucocorticoid treatment.

The physiological consequences of CBG changes in the response of the PA axis to a severe stressor such as IMO are not known. From the classical point of view of steroid action, a decrease in CBG levels would increase the free fraction of circulating corticosterone, with the subsequent increase in the efficacy of negative feedback exerted by corticosterone. However, reduced CBG levels might also increase the biological activity of circulating glucocorticoids for several hours or days *(16)*, thereby increasing the pathological potential of IMO beyond that assumed on the basis of total circulating corticosterone levels. Nevertheless, it can be speculated that the impact of free glucocorticoid fraction on negative feedback mechanisms might be higher than on other biological activities. Therefore, this decrease in CBG might contribute more to shorten PA activation than to other glucocorticoid-related biological effects.

In summary, the present data indicate that acute stress-induced inhibition of CBG is found only by exposure to severe stressors and after a lag period of at least 6 h. The inhibition is proportional to the period of exposure to the stressor and was eliminated by adrenalectomy, but not by RU486 administration, which suggests that stress-induced inhibition of CBG is dependent on the adrenal, but does not appear to be mediated by glucocorticoids acting through their classical type II receptors.

Materials and Methods

Male Sprague-Dawley rats 3–4 mo old were used. They were maintained 2–4 per cage in a controlled environment (lights on from 07.00 to 19.00 h, temperature 22°C) for at least 1 wk before starting the experiments. Food and water were provided ad libitum. The experiments were approved

by the Ethical Committee of the Universidad Autónoma de Barcelona.

In Exp. 1A and 1B, the effect of 1, 6, and 24 h of IMO stress on serum CBG levels was studied. To that end, the rats were either left undisturbed in the animal room (controls) or immobilized in woodboards as described *(4)*. One- (Exp. 1A) and 24 h-stressed (Exp. 1B) rats were killed along with control rats between 10.00 and 11.00 h. Since 24-h IMO rats had no access to food and water, an additional group of rats that remained undisturbed in the animal room with no access to food and water was also included. Six-hour IMO rats were killed along appropriate control rats between 15.30 and 16.00 h.

In Exp. 2, the effect of removing the adrenal gland on stress-induced changes in CBG was studied. Three groups of rats were used:

1. Intact control, rats that were left undisturbed;
2. ADX, rats that were anesthesized with an ip dosis of pentobarbital (45 mg/kg) and adrenalectomized by dorsal approach;
3. SHAM-ADX, rats that were treated as ADX rats, the adrenal being exposed, but not removed.

The ADX rats received saline (0.9 % NaCl) instead of tap water. Three days after surgery, morning blood samples from the three groups were obtained by retro-orbital venipuncture under slight ether anesthesia (prestress values). After that, the intact rats were left undisturbed until killed 18 h later (and served as control for the effect of stress associated to blood sampling), whereas ADX and SHAM-ADX groups were subjected to 18 h of IMO and then killed along the intact rats. Killing procedure was carried out between 10.00 and 11.00 h. Animals having noncomplete ADX were excluded. A period of exposure to IMO shorter than 24 h was used because of the special sensitivity of ADX rats to stress.

In Exp. 3, the putative involvement of glucocorticoids on stress-induced changes of CBG levels was studied by administering the glucocorticoid receptor antagonist RU486. Rats were given (p.o.) vehicle (0.2% polysorbate 80, 0.25% carboxymethylcellulose) or RU486 (100 mg/kg in a volume of 5 mL/kg). Two hours later, some vehicle and RU-treated rats were subjected to IMO for 18 h.

In Exp. 4, the effect of restraint and IMO on serum corticosterone and CBG levels was compared. The putative relevance of the length of exposure to stress and the time elapsed between the end of stressor exposure and the blood sampling was also evaluated. For the first objective, the animals were restrained or immobilized for 2, 6, or 24 h and killed along appropriate controls, the stress procedure being started in the morning at 10.00 h. For the second objective the effect of different periods of stress (IMO) combined with different resting periods was studied in parallel, in order to discriminate between the need for a continuous exposure to stress, and the time needed for a change of CBG levels to occur. Thus, some rats were stressed for 2 h, returned to their home cages and allowed to rest for 4 h, and then killed; these rats can, therefore, be compared to those continuously stressed for 6 h. Similarly, other rats were immobilized either 2 or 6 h, returned to their homecages, and allowed to rest for either 22 or 18 h, respectively; these rats can then be compared to those continuously stressed for 24 h.

Killing Procedure and Analysis

The rats were quickly killed by decapitation in a room adjacent to the animal house and the stress room. Unless otherwise stated, the rats were killed during the morning between 09.00 and 12.00 h. Trunk blood was collected, maintained, and centrifuged at 4°C, and the serum obtained frozen at –20°C. The samples selected to measure CBG levels were lyophilized and sent to the United States to be determined by a homologous RIA *(28)*. It was found that all these procedures did not alter CBG levels. In this assay the minimal detectable amount was 4.5 fmol, the intra-assay CV was 10%, and interassay CV ranged from 9.6 to 15.4%. In the last experiment, CBG levels were determined by a binding assay in stripped serum, using 3H-corticosterone (Amersham) with a specific activity of 82 Ci/mmol. One hundred microliters of diluted (1:200) serum were incubated with 20 n*M* 3H-corticosterone in a final volume assay of 500 µL. Nonspecific binding was measured using 1 m*M* corticosterone. The assay buffer was 0.01*M* phosphate (pH = 8.2) containing 0.9% NaCl and 1% gelatine. The incubation lasted for 30 min at 37°C and 15 min at 4°C. Five hundred microliters of a solution containing dextran T70 (0.1%) and charcoal (1%) were added, and 10 min later the tubes were centrifuged at 4°C for 15 min. The results from a pilot experiment showed that a 28% dissociation was produced with regard to time 0 and the CBG values were accordingly corrected. Serum corticosterone was determined by a direct RIA, as described previously *(29)*.

Statistical Analysis

The statistical significance of the results was analyzed by the Student's *t*-test when two means were compared, with one-way ANOVA followed by the Student–Newman–Keuls (SNK, $p < 0.05$) test of posthoc comparisons when more than two means were compared; and finally, with two-way ANOVA when the influence of two factors was studied.

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