Direct evidence of acute stress-induced facilitation of ACTH response to subsequent stress in rats

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Direct evidence of acute stress-induced facilitation of ACTH response to subsequent stress in rats. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R863–R868, 1999.—To determine the role of glucocorticoids in the appearance of the facilitatory effect of stress on the ACTH response to a subsequent stress, sham-operated (Sham) rats and rats adrenalectomized (ADX) and supplemented with 50 mg/l corticosterone (B) in the drinking saline (ADX + B) were subjected to 1 min of immobilization stress (Imo) four consecutive times with an interstressor interval of 90 min. Sham rats showed a similar pattern of ACTH response to the first and fourth exposures to Imo. ADX + B rats showed an exaggerated ACTH response to the fourth Imo, despite higher stress levels than those observed before the first Imo. In another experiment, no facilitatory effect of previous stress on ACTH response was found in ADX rats, but supplementation with B in the drinking saline for 1 wk resulted in facilitation of the ACTH response. We conclude that repeated exposure to a short-time stress induces a facilitatory effect on the ACTH response that is uncovered by eliminating stress-induced glucocorticoid release but needs B doses resulting in approximately basal circulating glucocorticoid levels to be induced or expressed.

hypothalamic-pituitary-adrenal axis; stress; facilitation; adrenalectomy

STRESSFUL STIMULI are able to activate the hypothalamic-pituitary-adrenocortical (HPA) axis in all vertebrates, acting mainly through the central nervous system. The activity of the HPA axis is restricted by the negative feedback exerted by glucocorticoids at various areas known to have corticosteroid receptors and to be involved in the control of the HPA axis: hippocampus, hypothalamus, and pituitary (10). Accordingly, exogenous administration of glucocorticoids is able to reduce HPA activation caused by a stressor. However, as early as 1973, Dallman and J ones (9) observed that high blood glucocorticoid levels achieved by exposure to a stressor instead of by exogenous administration did not block the HPA response to a subsequent stressor. It has been hypothesized that the lack of an inhibitory effect of glucocorticoids released by previous stress was due to the fact that stress would induce a facilitation of the HPA axis that counteracts the inhibitory action of glucocorticoids (8). This hypothesis is very attractive and well accepted by researchers in the field of the HPA response and stress, but there are many aspects to be clarified.

First, the concept of stress-induced facilitation of the HPA axis has been extended to chronic stress conditions (8). When chronically stressed animals face a novel acute stressor, a greater ACTH response has sometimes been found, supporting the idea that, under certain conditions, chronic stress exposure might facilitate the subsequent ACTH response to stress. In this regard, Hauger et al. (14) found a progressive increase of the ACTH response to ether during chronic intermittent immobilization. Scribner et al. (24), using streptozotocin-induced diabetes in rats, observed an exaggerated ACTH and corticosterone (B) response to the acute stress provoked by histamine injection. In our laboratory we have also observed an exaggerated ACTH response to a novel stressor in chronically immobilized, adrenalectomized (ADX) rats, suggesting that stress-induced glucocorticoid release might be masking the facilitatory effect of previous stress (21). However, chronic stress involves long-lasting exposure to stressors, and therefore it may induce changes in the HPA axis that might not be similar, at least in part, to those observed after acute stress.

Second, the HPA response to stress is sometimes, but not always, maintained in animals previously stressed in the preceding hours. Thus it has been reported that the HPA response is maintained (or even increased) when the stressors are mild (18) or when the exposure to them is brief and the interstressor interval is relatively long (7, 9, 11, 12, 19, 28). After exposure to stronger stressors or with shorter interstressor intervals, a reduced response to the second stress has been observed (5, 13, 15, 19). These contradictory results on the effect of acute stress on the HPA response to a subsequent stress might derive from the fact that the HPA response to stress is the result of a subtle balance between the negative feedback exerted by stress-induced glucocorticoid release and the stress-induced facilitation.

Third, the only direct proof of acute stress-induced facilitation has been obtained by Akana and Dallman (3) using rats in which the B response to stress was inhibited by drugs interfering with glucocorticoid biosynthesis. Their data are not easy to interpret, in that the rats, when previously subjected to acute stress at lights-on but tested only at lights-out, showed no facilitation of the ACTH response, whereas when stressed at lights-out but tested at lights-on, they did show facilitation. It is therefore unclear whether the absence of facilitation caused by exposure to stress at lights-on was due to the lack of expression of facilitation in the dark period of the circadian cycle, as...
proposed by Akana and Dallman, or to a lower efficacy of facilitatory mechanisms in animals stressed at lights-on.

The aim of the present work was to demonstrate directly the existence of facilitation of the ACTH response in an acutely repeated stress model in the rat. To avoid the possible interference of stress-induced B feedback on the facilitatory effect of stress, we have used ADX animals supplemented with B in the drinking saline to simulate diurnal basal B levels and prevent its elevation in response to stress. It was found that previous exposure for 1 h to a severe stressor such as immobilization (Imo) did not induce facilitation of the HPA response to a further stress in ADX rats or in ADX rats supplemented with B in the drinking saline (20a). On the contrary, a blunted ACTH response to the second stressor was found. We then thought that facilitation might be apparent with use of brief and repeated exposures to a stressor and followed the protocol previously used by De Souza and Van Loon (11). With this protocol we have obtained direct evidence for acute stress-induced facilitation of the hypothalamus-corticotrope axis in ADX rats maintained with low-dose B in the drinking saline.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, obtained from the breeding center of our university, were 45 days old when they were used. They were kept two per cage in standard conditions of photoperiod (lights-on from 0730 to 1930) and temperature (22 ± 1°C) for ≥1 wk before and throughout the experiments. They weighed 295 ± 31 g at the beginning of the experiments. Rats had free access to food and water (or saline). The protocols were approved by the Committee of Ethics of the Universitat Autònoma de Barcelona.

Surgery and stress procedure. One week before starting the experiments the animals were anesthetized with diethyl ether and subjected to bilateral ADX by the dorsolateral approach or to ADX simulation (Sham). After surgery, ADX rats were given 0.9% saline or 0.9% saline supplemented with 50 mg/l B (ADX + B); the hormone (Sigma Chemical) was dissolved first in ethanol (50 mg/4 ml) and then in 1 liter of saline. Fresh solutions were prepared every 2 days. Circulating B levels were measured, and the possible presence of remnant adrenal tissue was evaluated at autopsy. Those animals not subjected to total ADX were eliminated from the statistical analysis. Rats were stressed by Imo, according to Kvetnansky and Mikulaj (16). Briefly, rats were immobilized on a wooden board in a prone position by taping their four limbs to metal mounts. Head movements were restricted by connecting B levels were measured, and the possible presence of remnant adrenal tissue was evaluated at autopsy. Those animals not subjected to total ADX were eliminated from the statistical analysis. Rats were stressed by Imo, according to Kvetnansky and Mikulaj (16). Briefly, rats were immobilized on a wooden board in a prone position by taping their four limbs to metal mounts. Head movements were restricted by connecting

RESULTS

Experiment 1. The ACTH response to repeated Imo stress was studied in Sham and ADX + B rats. Basal ACTH levels were similar in Sham and ADX + B rats (Fig. 1), suggesting that B availability in the water was apparently enough to maintain a normal basal corticosterone activity in ADX rats. One-way ANOVA revealed a significant time effect on plasma ACTH levels in Sham and ADX + B rats (P < 0.001 in all cases). Post hoc comparisons with the paired t-test revealed that the first and fourth exposures to Imo increased plasma ACTH levels in Sham and ADX + B rats compared with their respective prestress values (P ≤ 0.002 in all cases). Previous exposures to Imo resulted in higher ACTH levels before exposure to the fourth Imo in ADX + B (P < 0.001 vs. initial values before the first Imo) but not in Sham rats. When the net ACTH response to repeated Imo stress was studied in Sham and ADX + B rats. Basal ACTH levels were similar in Sham and ADX + B rats (Fig. 1), suggesting that B availability in the water was apparently enough to maintain a normal basal corticosterone activity in ADX rats. One-way ANOVA revealed a significant time effect on plasma ACTH levels in Sham and ADX + B rats (P < 0.001 in all cases). Post hoc comparisons with the paired t-test revealed that the first and fourth exposures to Imo increased plasma ACTH levels in Sham and ADX + B rats compared with their respective prestress values (P ≤ 0.002 in all cases). Previous exposures to Imo resulted in higher ACTH levels before exposure to the fourth Imo in ADX + B (P < 0.001 vs. initial values before the first Imo) but not in Sham rats. When the net ACTH response to repeated Imo stress was studied in Sham and ADX + B rats. Basal ACTH levels were similar in Sham and ADX + B rats (Fig. 1), suggesting that B availability in the water was apparently enough to maintain a normal basal corticosterone activity in ADX rats. One-way ANOVA revealed a significant time effect on plasma ACTH levels in Sham and ADX + B rats (P < 0.001 in all cases). Post hoc comparisons with the paired t-test revealed that the first and fourth exposures to Imo increased plasma ACTH levels in Sham and ADX + B rats compared with their respective prestress values (P ≤ 0.002 in all cases). Previous exposures to Imo resulted in higher ACTH levels before exposure to the fourth Imo in ADX + B (P < 0.001 vs. initial values before the first Imo) but not in Sham rats.
increase was considered (response to stress after subtraction of appropriate prestress levels), paired t-test revealed no differences between the first and fourth stress exposures in Sham rats. In contrast, in ADX + B rats the net ACTH increase after the fourth stress doubled approximately that seen after the first stress ($P < 0.05$).

Plasma B levels are shown in Table 1 for Sham and ADX + B rats. The one-way ANOVA of plasma B levels in Sham rats revealed a significant time effect ($P < 0.001$). Post hoc comparisons revealed a strong B response to the first and fourth Imo exposures ($P < 0.001$ in both cases) and higher B levels before exposure to the fourth than to the first Imo ($P < 0.001$). The net increase in B was similar after the first and fourth Imo exposures. No significant time effect on plasma B levels in ADX + B rats was observed, but B levels were higher than in nonstressed Sham rats ($P < 0.001$), suggesting that corticosterone replacement was above that needed to maintain strict diurnal basal levels.

Experiment 2. The effect of repeated exposure to Imo was studied in ADX rats. The one-way ANOVA revealed a significant time effect on ACTH levels ($P < 0.006$). Paired t-test revealed an increase in plasma ACTH levels in response to the first Imo ($P < 0.01$ vs. basal levels before any Imo), higher ACTH levels just before the fourth Imo than before the first Imo ($P < 0.03$ vs basal levels before any Imo), and no further response to the fourth Imo (Fig. 2A).

When the same ADX animals were given B in the drinking saline for 1 wk and studied again, a significant time effect was observed (Fig. 2B). Further comparisons showed that, as in experiment 1, 1) plasma ACTH levels increased in response to the first and the fourth Imo exposures ($P < 0.05$ and $P < 0.005$ vs. respective basal values), 2) ACTH levels were higher before the exposure to the fourth than to the first Imo ($P < 0.05$ vs. basal levels before the 1st Imo), and 3) the net ACTH increase in response to stress after the fourth Imo was twofold that after the first Imo ($P < 0.02$).

**DISCUSSION**

In the present work we aimed to study the possible facilitatory effect of repeated acute stress on the subsequent ACTH response to a further stress. We have used rats with full adrenocortical response to stress (Sham) and ADX rats, which in some cases were supplemented with B in the drinking saline to maintain approximately diurnal basal B levels. They were exposed four times to Imo for 1 min, with an interstressor interval of 90 min. In choosing this experimental approach, we have taken into account that 1) previous data in the literature suggested the existence of facilitation also with use of short time stressors (11, 18), 2) Imo is a high-intensity stressor capable of eliciting a robust ACTH response (4), and 3) a relatively quick return of ACTH to basal levels was expected because of the very short time of exposure to the stressor.

The results of experiment 1 showed that in Sham rats ACTH levels before exposure to the fourth Imo were similar to those observed before the first Imo. In addition, the ACTH response to the fourth Imo was similar to the response to the first Imo. This indicates that in adrenal-intact rats acutely repeated Imo is not able to induce any facilitation of the ACTH response to the same stressor and that an interstressor interval of 90 min is long enough to allow a total recovery of basal

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**Table 1. Plasma B levels in acute repeatedly stressed rats of experiment 1**

<table>
<thead>
<tr>
<th></th>
<th>Imo 1</th>
<th>0 min</th>
<th>20 min</th>
<th>Imo 4</th>
<th>0 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham 8</td>
<td>1.0 ± 0.6</td>
<td>36.9 ± 4.0*</td>
<td>11.7 ± 3.5†</td>
<td>51.6 ± 4.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX + B 10</td>
<td>3.9 ± 1.1‡</td>
<td>2.8 ± 0.4</td>
<td>4.4 ± 1.3</td>
<td>3.4 ± 0.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. ADX, adrenalectomized; Imo 1 and Imo 4, 1st and 4th exposures to immobilization. *$P < 0.001$ vs. respective 0 min value; †$P < 0.001$ vs. 0 min Imo 1. ‡$P < 0.001$ vs. Sham.
ACTH levels. At the same time, the persistence of the ACTH response to the fourth Imo in Sham rats, with ACTH levels similar to those achieved after the first exposure, points out that no short-term habituation of the HPA response to stress occurs in this experimental model, which might have made the interpretation of data in ADX + B rats more complex.

Basal ACTH levels of ADX + B rats just before the first stress exposure were comparable to those observed in Sham rats, which proves that B levels achieved in blood in these animals were able to restrain ACTH hypersecretion caused by ADX. However, the pattern of ACTH response to repeated Imo in ADX + B rats was different from that in adrenal-intact rats. First, ADX + B rats showed elevated ACTH levels before exposure to the fourth stress exposure compared with their levels before any stress or with Sham rats, suggesting that stress-induced ACTH secretion declines more slowly in ADX + B animals, likely because of the absence of negative corticosterone feedback through type II corticosteroid receptors (25). Despite high ACTH levels just after the fourth Imo, the ACTH response to the stressor doubled that observed after the first Imo. Therefore, in the absence of stress-induced B release, facilitation of the ACTH response to an acute stressor appears to occur in ADX + B rats, directly demonstrating the existence of a facilitatory effect of prior stress on the ACTH response to a subsequent stress. These data confirm the results by Akana et al. (3) using rats with a pharmacological blockade of stress-induced B release and their suggestion that stress at lights-on is able to activate facilitatory mechanisms within the central nervous system that are not likely to be expressed at lights-out.

We did not know whether the maintenance of ADX animals with low B levels and the normal prestress activity of the HPA axis were important in expression of facilitation. We thus decided to directly prove it by using the same protocol in ADX animals without B replacement. The expected high basal ACTH levels due to the absence of circulating B and a consistent ACTH response 20 min after the first exposure to Imo were found. The ACTH levels just before the fourth exposure of ADX rats to Imo were comparable to those observed 20 min after the first stress, and no further increase was observed in response to the fourth Imo. The lack of negative B feedback was presumably responsible for these high “basal” ACTH levels before the last exposure to Imo. Unrestrained ACTH release from the pituitary was not balanced by an adequate feedback input, and thus return of ACTH to normal levels took place very slowly. The absence of an ACTH response to the fourth stress appears to be, at first, surprising. However, several hypothetical mechanisms might explain the present results.

First, ACTH levels of ADX rats before exposure to the fourth Imo might be so high that no further increase might be possible. This does not appear to be the case, since plasma ACTH levels achieved after corticotropin-releasing factor (CRF) administration is considerably higher than those found in the present experiment (20a). Second, there is evidence that ACTH can exert a negative feedback on the hypothalamus-corticotrope axis (29), and more recent studies have shown an inhibitory action of ACTH and other proopiomelanocortin-derived peptides on in vitro hypothalamic CRF release and in vivo CRF mRNA in the paraventricular nucleus (6, 20, 23, 26). If these feedback mechanisms were actually effective in inhibiting hypothalamic stimulatory inputs to corticotropes, ADX rats could not respond to the fourth stress in our experimental paradigm because of the negative feedback exerted by ACTH released over the preceding hours. Finally, after completion of the present experiments, two laboratories reported very interesting data concerning the role of B in stress-induced facilitation of the hypothalamus-corticotrope response to stress. Tanimura and Watts (27) reported a defective CRF response to a sustained acute stress in ADX rats that was restored by low-corticosterone pellets, suggesting involvement of type I corticosteroid receptors. Akana and Dallman (2) published evidence that higher plasma B levels might be necessary for chronic stress-induced facilitation of the ACTH response to a novel stressor, in that a greater ACTH response to restraint was observed in chronic cold than in control rats in those ADX rats maintained with high-dose B pellets but not in those with lower-dose B pellets. The critical role of B in acute stress-induced facilitation was confirmed when the ADX animals were supplemented with B in the drinking solution for 1 wk.

It could be argued that high ACTH levels before the fourth exposure to Imo of ADX + B rats and the higher response before the first exposure could have been due to the different time of day at which the rats were exposed to the first and the fourth stress, that is, to circadian rhythms in basal and stress levels of ACTH in ADX + B rats. Although Akana et al. (1) observed an amplification of the circadian rhythm of basal ACTH levels in ADX rats implanted with low-B pellets, such an effect was observed at near lights-out, and our experiments were finished before 1400. In addition, such an amplification was not observed in the ACTH response to stress. Therefore, it is unlikely that this factor could be responsible for the differences between the first and the fourth exposure to Imo in ADX + B rats, although this merits direct testing. A contribution of changes in B levels throughout the experiment was also unlikely, inasmuch as plasma B levels were quite well maintained. Because rats had free access to saline in the interstress intervals, the maintenance of plasma B levels over the course of the experiment suggests that rats were taking sufficient saline solution to maintain morning B levels. In fact, a small but significant amount of saline is taken by ADX rats over the morning hours (unpublished data). In addition, a possibility remains that stress-induced B response to a short-time stress induces a facilitatory effect on the hypothalamus-corticotrope axis, which

In summary, the present results show that repeated exposure to a short-time stress induces a facilitatory effect on the hypothalamus-corticotrope axis, which
proves that the system is not only fully responsive to a subsequent stressor but that the ACTH response is exacerbated. The fact that a facilitatory effect of stress is not evident in ADX rats but appeared when they were given B in the drinking saline suggests that B is necessary to induce (or permit) facilitation of the hypothalamus-corticotrope axis.

**Perspectives**

The hypothesis of stress-induced facilitation of the HPA axis originally proposed by Dallman and J ones in 1973 (9) has greatly influenced the theoretical view of the stress field. Briefly, the authors suggested that corticosterone released during stress did not have the expected inhibitory effect on a subsequent activation of the HPA axis, because previous stress facilitated the central mechanisms controlling the HPA axis, thus overcoming glucocorticoid negative feedback. Although it appears that chronic stress potentially activates the HPA axis at various levels and, therefore, facilitates in some way the activity of the HPA axis, direct proof of acute stress-induced facilitation of the HPA axis is scarce. The present results demonstrate that brief and repeated exposure to stress actually facilitates the ACTH response to the same stressor and that this facilitation is masked by stress-induced corticosterone release. However, in accordance with some recent studies, approximately normal circulating corticosterone levels are necessary to induce facilitation, so corticosteroid type I receptors are probably involved. It appears that functional integrity of the HPA axis is exquisitely controlled by the concerted action of type I and II corticosteroid receptors.

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