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Crowding Stress and Adrenal Mitochondrial 11β-Hydroxylation in Vitro[1, 2] (39584)

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A variety of stressful environmental conditions are known to stimulate the activity of the hypothalamic-pituitary-adrenal complex. An increase in the population density of animals in natural as well as in laboratory conditions leads to adrenal hypertrophy, increased secretion of corticosteroids, as well as other responses associated with elevated release of ACTH (1-5). Slices of adrenal glands isolated from animals under situations of social or population density stress show enhanced corticosteroid release (2, 3). ACTH administration *in vivo* causes an increase in the capacity for 11β-hydroxylation by adrenal mitochondria *in vitro* (6, 7). Physiological studies dealing with crowding stress are hampered by the complexities involving quantification of the stressor and adaptive responses of the adrenal in terms of blood corticosterone determinations (8). Since adrenal mitochondrial 11β-hydroxylase activity can serve as an index of ACTH stimulation, measurement of this reaction *in vitro* offers a means of evaluating a stress response. The objective of the present investigation was to study the effects of various crowding densities in rats on the *in vitro* capacity of adrenal mitochondria to carry out the 11β-hydroxylation reaction.

Materials and methods. Male Holtzman rats, initial body weight 150-170 g, were housed in wire mesh cages and fed on Purina laboratory chow and tap water *ad libitum*. The rats were maintained at 22-23° on a 14-hr light/10-hr dark cycle. The normal control rats were provided 110 cm^2 of floor space per rat. For crowding, the animals were housed at 50 or 30 cm^2 of floor space per rat. Animals were kept under these conditions for 3 weeks before adrenals were obtained.

Since the hypothesis to be tested pertained to alterations in mitochondrial 11 β-hydroxylation in response to stress-associated elevation of endogenous ACTH, it was appropriate to validate the experimental procedure by employing hypophysectomized and ACTH-treated rats. Male rats were hypophysectomized through the intraaural approach using a Hoffman-Reiter stereotaxic instrument to position the animal. Adrenal glands were obtained from the hypophysectomized animals and from controls on Days 1, 5, and 10 following surgery. Exogenous ACTH (2 or 10 IU/rat; ACTHAR, Armour) was injected sc into intact male rats. Control animals were injected with isotonic saline. Adrenal glands were obtained 24 hr following injection. Both the removal of the adrenal glands and the subsequent incubation of mitochondria were carried out at approximately the same time of day.

Animals were decapitated, adrenal glands were removed and placed on filter paper moistened with 0.25 *M* sucrose in a cold chamber. In the hypophysectomized rats, the sella turcica area was examined following decapitation. In those cases where removal of the pituitary gland was considered to be incomplete, the adrenal glands were discarded. Glands were cleaned of adhering fat, weighed, and homogenized in 0.25 *M* sucrose containing 20 m*M* triethanolamine (TRA) buffer, pH 7.4. Mitochondria were obtained by the method of Peron and McCarthy (9). For each *in vitro* test adrenal mitochondria were prepared from 5 to 10 experimental rats and from a similar number of respective controls.

Aliquots of the mitochondrial fraction, suspended in the sucrose-TRA buffer mixture, were incubated in a media containing 20 m*M* TRA, pH 7.4, 50 m*M* nicotinamide, 180 nmole deoxycorticosterone (DOC),

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[2] Nomenclature of chemicals for which trivial names or abbreviations are used: deoxycorticosterone, DOC, 21-hydroxy-4-pregnene-3,20-dione; corticosterone, B, 11β, 21-dihydroxy-4-pregnene-3,20-dione; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

and either 10 m*M* isocitrate, succinate malate, or α-ketogluterate as oxidizable substrate to support the reaction (9, 10). In some cases, 5 m*M* phosphate was included in the incubations containing 10 m*M* malate (11). The final volume of the incubate was brought to 2.0 ml with 0.154 *M* KCl. The reactions were carried out in a Dubnoff metabolic incubator at 37° under oxygen for 15 min and terminated by freezing the media.

The corticosterone (B) content of the incubation media was analyzed using sulfuric acid fluorescence technique (12). Protein content was determined by the method of Lowry *et al* (13). The results are expressed as nanomoles B formed per milligram of mitochondrial protein in 15 min. In each case, data presented are based on separate measurements obtained from two or three different groups of animals.

Results. Adrenal mitochondria obtained from rats 5 or 10 days following hypophysectomy showed the expected reduction in capacity for 11β-hydroxylation of DOC *in vitro* (Fig. 1). The B production on Day 5 was more than 50% below the control value. On the first day following surgery, however, the corticosteroid conversion was consistently greater in the operated than in control rats.

Compared to controls, adrenal mitochondria obtained from rats 24 hr after ACTH injection showed an increased capacity to carry out 11β-hydroxylation in the presence of oxidizable intermediates (Fig. 2). Administration of 10 U of ACTH resulted in a two- to fourfold increase in B formation by mitochondria in the presence of succinate, α-ketoglutarate, or malate.

The data on the effects of crowding on substrate-supported mitochondrial 11β-hydroxylation are presented in Fig. 3. In groups with 50 cm² of floor space per rat, mitochondria showed little or no variation from the controls. A further reduction to 30 cm² of floor space per rat resulted in a marked increase in DOC conversion with each of the substrates tested.

In order to evaluate the duration of the mitochondrial response to crowding some rats housed at 30 cm² of floor space per rat for 3 weeks were returned to the control conditions, i.e., 110 cm² of floor space per rat. Following 3 weeks under the normal conditions the activity of the adrenal mitochondria were compared to those maintained for a period of 6 weeks under the control conditions. The capacity for *in vitro*

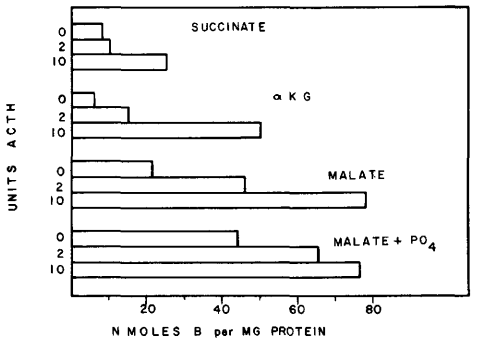

FIG. 2. *In vitro* hydroxylation of DOC by adrenal mitochondria prepared from intact male rats 24 hr after a single sc injection of ACTH.

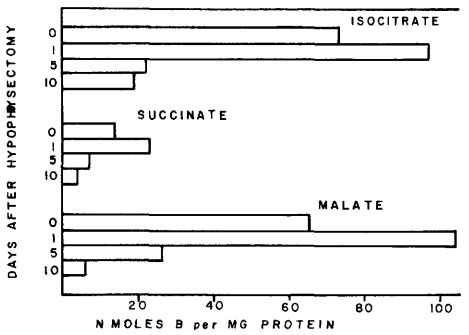

FIG. 1. The effect of time after hypophysectomy on *in vitro* hydroxylation of deoxycorticosterone by rat adrenal mitochondria. Values for Day 0 represent preparation from intact rats.

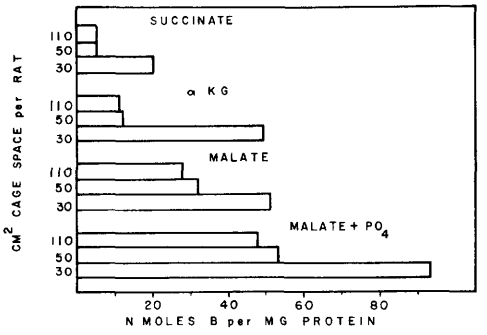

FIG. 3. *In vitro* hydroxylation of DOC by adrenal mitochondria prepared from intact male rats after housing for 3 weeks under the conditions indicated in the figure.

11β-hydroxylation in the crowded rats 3 weeks after transfer to control conditions remained elevated at levels similar to those obtained immediately at the termination of 3 weeks of crowding (as in Fig. 3). It appears that the effects of severe crowding result in the elevation of the capacity of adrenal mitochondrial for hydroxylation of DOC and the response persists for a period following the removal of the stressor.

Discussion. The results of this investigation demonstrate that the stressful severity of two different housing conditions can be differentiated on the basis of *in vitro* capacity of adrenal mitochondria to carry out the 11β-hydroxylation reaction. Studies of Christian and co-workers, as well as work by others (1–5), have amply demonstrated that crowding produces alterations in adrenal activity. Increases in the population density of previously individually housed mice resulted in an increase in adrenal weight, in the width of zona faciculata and in *in vitro* corticosteroid production by the adrenal sections (see 1 and 2). These responses represent the generalized reaction of the organisms to stress. Within a population of a given density the potentially stressful stimuli may be due to the pressure of numbers as well as the member interactions (1). Whereas the effect of the former may be felt by all members of the population, the influence of the latter is, however, more selective. Social interactions between the members of a population are known to cause a greater degree of stress in the submissive than in the dominant members (1, 14). An intragroup variation in the degree of stressful reaction is, therefore, to be expected.

In the present study, the adrenals from the rats within the same cage were processed together. The measurements, therefore, represent the average of the cumulative stress response of a given group. Based on the group response, it is apparent that the decrease in housing space from 110 cm^2/rat to 50 cm^2 did not result in any significant alteration in the hydroxylation response at the end of the 3-week period. A further reduction to 30 cm^2 of floor space per rat led to an elevation in the 11β-hydroxylation reaction, indicative of a stress response. It should be pointed out that in the group held at 50 cm^2/rat, we cannot exclude the possibility of an earlier or transient stress response followed by a normalization by the end of the 3-week period. If a transient response did occur in the 50-cm^2 groups, then the more dense housing conditions (30 cm^2/rat) could be viewed as increasing the severity and/or duration of the stress response. It is of interest to note that in mice, a more aggressive species than the rat, increase in population to a minor extent causes elevation in stressful responses (1, 2, 4, 5).

The levels of 11β-hydroxylation obtained in the presence of oxidizable substrates (see Figs. 1–3) are comparable to those reported elsewhere (7, 9–11). The 11β-hydroxylation supported by an oxidizable substrate is related to its efficiency to generate intramitochondrial NADPH, with $NADP^+$-linked oxidation of isocitrate being most effective in the rat. On the basis of the relative levels of DOC hydroxylated in the presence of various substrates it was possible to correlate the experimental regimens with alteration in 11β-hydroxylase activity.

The 11β-hydroxylase, an enzyme specific to adrenal mitochondria, catalyzes the final step in glucocorticoid formation, i.e., the conversion of DOC into B. ACTH exerts its trophic action in stimulating the first step in corticosteroidogenesis involving the conversion of cholesterol to pregnenolone. The rate of this reaction significantly increases within 10 min of ether stress (15). The 11β-hydroxylase activity also increases following ACTH (6, 7, 16) and decreases after hypophysectomy (17; Fig. 1). A single injection of ACTH elevates the conversion of DOC into B within 30 to 60 min and the effect can be seen after 24 hr (7; Fig. 2). It would thus appear that the elevation of 11β-hydroxylase activity, which is consistently noted on the first day following hypophysectomy (Fig. 1), is due to the ACTH release in response to the stress of etherization and surgery. However, the levels of 11β-hydroxylation 24 hr postsurgery tend to be higher than those 24 hr after administration of ACTH (Figs. 1 and 2). The nature of factors, released in addition to ACTH, responsible for this difference is uncertain.

The 11β-hydroxylase shows a peculiar time-associated activity after hypophysectomy (Fig. 1). After surgery this enzyme

activity remains at elevated levels at 24 hr. In contrast, it is well established that adrenal secretion decreases within minutes while adrenal sensitivity to exogenous ACTH diminishes within a few hours following hypophysectomy. The decrease in 11β-hydroxylation to less than 50% of the controls 5 days following surgery (Fig. 1) is consistent with the previously determined half-life of 4 days for this enzyme (17). The activity of the 11β-hydroxylase thus reflects levels of ACTH to which the adrenal gland had been previously exposed and likewise can be used as a measure of the severity of a stressor.

Relatively little is known concerning the physiological changes which persist following removal of a stressor. Our attempt to reverse crowding stress by returning the animals to the normal housing conditions did not result in a decline in 11β-hydroxylation. Since the half-life of the 11β-hydroxylase is 4 days (17), the high levels of DOC hydroxylation 3 weeks after the termination of crowding stress suggest the possible persistence of ACTH stimulation. The nature of the mechanism responsible for this response is obscure. It is known that prolonged administration of ACTH or chronic exposure to stress is associated with several pathological conditions which include cardiac hypertrophy, glomerulonephritis, and liver damage (1, 3, 18–21). It is conceivable that pathological conditions may themselves be associated with persistence of physiological changes in the post-stress period.

Quantitative ultrastructural studies have been undertaken on cardiac myocytes of rats in stress and in the post-stress period (McCarthy and Sohal, unpublished observations). Some cardiac changes, such as mitochondrial volume, lipid accumulation, and separation of intercalated disks, were more severe 3 weeks after removal from stress than immediately after crowding itself. Further studies are needed to elucidate the relationship between such changes and the persistence of apparent adrenal hyperactivity.

Summary. Adrenal mitochondria were obtained from rats subjected to two different crowding regiments in order to test the *in vitro* capacity for 11β-hydroxylation (11β-OH) as an index of stress. Compared to controls, 110 cm² of floor space per rat, crowding at 30 cm²/rat markedly increased 11β-OH. Little change occurred when rats were held at 50 cm²/animal. High levels of 11β-OH persisted 3 weeks after the termination of severe crowding. This apparent failure to reverse crowding stress is viewed in terms of pathologic changes that appear to accompany crowding stress. On the basis of measuring 11β-OH capacity, it is possible to differentiate between the stressful effects of the housing conditions.

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