Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner

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Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. The adrenal gland is an essential stress-responsive organ that is part of both the hypothalamic-pituitary-adrenal axis and the sympatho-adrenomedullary system. Chronic stress exposure commonly increases adrenal weight, but it is not known to what extent this growth is due to cellular hyperplasia or hypertrophy and whether it is subregion specific. Moreover, it is not clear whether increased production of adrenal glucocorticoid after chronic stress is due to increased sensitivity to adrenocorticotrophic hormone (ACTH) vs. increased maximal output. The present studies use a 14-day chronic variable stress (CVS) paradigm in adult male rats to assess the effects of chronic stress on adrenal growth and corticosterone steroidogenesis. Exogenous ACTH administration (0–895 ng/100 g body wt) to dexamethasone-blocked rats demonstrated that CVS increased maximal plasma and adrenal corticosterone responses to ACTH without affecting sensitivity. This enhanced function was associated with increased adrenal weight, DNA and RNA content, and RNA/DNA ratio after CVS, suggesting that both cellular hyperplasia and hypertrophy occurred. Unbiased stereological counting of cells labeled for Ki67 (cell division marker) or 4,6-diamidino-2-phenylindole (nuclear marker), combined with zone specific markers, showed that CVS induced hyperplasia in the outer zona fasciculata, hypertrophy in the inner zona fasciculata and medulla, and reduced cell size in the zona glomerulosa. Collectively, these results demonstrate that increased adrenal weight after CVS is due to hyperplasia and hypertrophy that occur in specific adrenal subregions and is associated with increased maximal corticosterone responses to ACTH. These chronic stress-induced changes in adrenal growth and function may have implications for patients with stress-related disorders.

Ki67; corticosterone; adrenocorticotrophic hormone; adrenal cortex; adrenal medulla

APPROPRIATE PHYSIOLOGICAL RESPONSES to stress are important for survival. The hypothalamic-pituitary-adrenocortical (HPA) axis and the sympatho-adrenomedullary axis are the primary systems that are responsible for the maintenance of homeostasis during stress, and the adrenal gland is an essential organ that is common to both systems. For the HPA axis (reviewed in Ref.37), hypophysiotropic neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete releasing hormones, such as corticotropin-releasing hormone (CRH) and vasopres sin, into the portal circulation of the median eminence. These releasing hormones act on the anterior pituitary to promote the secretion of adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH acts on the inner adrenal cortex (i.e., the zona fasciculata) to produce glucocorticoid hormones (e.g., corticosterone in rats and cortisol in humans). In addition, ACTH can stimulate the outer adrenal cortex (i.e., the zona glomerulosa) to produce adrenosterone (30) in concert with the renin-angiotensin system. For the sympatho-adrenomedullary system (reviewed in Ref. 35), neural activation of the sympathetic nervous system results in the “fight or flight” response, which includes activation of neurally-derived chromaffin cells in the adrenal medulla. Chromaffin cells release catecholamines and neuropeptide hormones into the systemic circulation. Collectively, the glucocorticoid and catecholamine hormones have complementary actions throughout the body, including energy mobilization and maintenance of blood pressure (35).

Although the stress responses described above are imperative for survival during acute stress, frequent or prolonged activation can change the functional tone of these systems. Previous work (36, 47, 57, 67) looking at the effects of chronic stress on the HPA axis has focused largely on characterizing chronic stress-induced brain alterations, such as increased expression of CRH and vasopressin in the PVN and decreased expression of glucocorticoid receptors in the hippocampus and PVN. This central focus has occurred in part because the observed brain changes resemble those that are believed to occur in some types of stress-related psychiatric disorders, such as depression and anxiety (6, 9, 22, 23, 39, 46). However, rats exposed to chronic stress often exhibit adrenal enlargement and increased basal plasma corticosterone despite normal plasma ACTH levels, suggesting that chronic stress also affects the peripheral limb of the HPA axis (31, 49, 58, 63, 84). Moreover, many patients with depression have increased basal plasma cortisol and enlarged adrenals (4, 15, 24, 25, 64, 71, 72, 77). Furthermore, increased glucocorticoid levels have been linked with the onset and severity of depression (22, 32), suggesting that alterations in peripheral HPA axis structure and function may also be clinically relevant.

The purpose of the present study is to use a chronic variable stress (CVS) paradigm in rats to characterize the effects of chronic stress on adrenal morphology and cortical function. Early in vivo work using one or two doses of ACTH suggests that adrenal responses to ACTH are increased after chronic stress (7, 8, 70); however, it is not known whether enhanced...
responsiveness is due to increased sensitivity to ACTH [e.g., lower half-maximal dose (ED50)] and/or elevated maximal output [e.g., higher efficacy or maximum binding capacity (Bmax)]. Also, it is not clear whether adrenal enlargement after chronic stress is due to cellular hypertrophy (increased cell size) and/or hyperplasia (increased number of cells) or whether these types of growth are restricted to specific subregions of the adrenal gland. The present work addresses the hypothesis that adrenal responses to ACTH (both sensitivity and efficacy) are augmented after chronic stress and that this increased responsiveness is associated with cellular hypertrophy and hyperplasia in the zona fasciculata.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (approximate starting weight of 275–300 g for experiment 1 and 300–325 g for experiment 2; Harlan, Indianapolis, IN) were used. Rats arrived ≥1 wk before the onset of experiments and were pair-housed on a 12:12-h light-dark cycle (0600–1800) with ad libitum access to normal rat chow and water. All procedures were approved by the University of Cincinnati Animal Care and Use Committee.

CVS. The CVS model was chosen because it induces many behavioral and biochemical characteristics that are similar to human depression, including anhedonia, reduced body weight, sleep disturbances, glucocorticoid hypersecretion, and increased brain CRH expression (16, 36, 82). For experiment 1, CVS consisted of twice daily exposure (at ~930 and 1500) to one of several stressors presented in an unpredictable order for 14 days. All CVS rats were given the same schedule of CVS stress exposure. Stressors included hypoxia (8% O2, 92% N2, 30 min), warm swim (28–31°C, 15 min), cold swim (16–18°C, 5 min), cold room (4°C, 1 h), shaker platform (100 rpm, 1 h), and restraint (30 min). As an additional stressor, CVS rats were occasionally (1–2 nights/wk) housed overnight in isolation (1 rat/cage) or crowding (6 rats/cage). Nonhandled rats were used as controls. Body weight was measured at the onset and midpoint of the CVS paradigm (days 1 and 8, respectively). The CVS protocol for experiment 2 was identical to that described above, except that restraint was not included.

Experiment 1. On the morning (0600) after completion of the CVS paradigm (day 15), rats (n = 36 CVS, n = 36 control) were weighed and given dexamethasone phosphate (400 μg in sterile saline, sc) to block release of endogenous ACTH. Two to six hours later, rats (n = 6/group) were given an exogenous dose of rat ACTH-(1–39) (0, 21.6, 43.2, 64.8, 86.5, or 865 ng/100 g body wt in 0.5% bovine serum albumin in phosphate buffered saline, pH 7.4; Sigma-Aldrich, St. Louis, MO), presented in a random order by an experimenter unaware of doses. Rats were killed by decapitation at 15 min after administration of ACTH, and trunk blood was collected for measurement of plasma ACTH and corticosterone. Adrenal glands were quickly removed, cleaned, and weighed. One adrenal from each rat (equal representation of left vs. right adrenals) was randomly selected for measurement of adrenal DNA and RNA content. The other adrenals were used for immunohistochemical assessment of adrenal cellular hyperplasia and hypertrophy. Thymus glands were also removed, cleaned, and weighed.

Hormone and osmolality measurements. Blood samples were centrifuged (3,000 g, 15 min, 4°C), and plasma was stored at −20°C until assayed. Plasma corticosterone levels were assessed by radioimmunoassay (RIA) kit (MP Biomedicals, Orangeburg, NY), as described previously (79), with an intra-assay coefficient of variation (CV) of 8.6% and an interassay CV of 13.6%. Adrenal corticosterone content was determined by homogenization in ethanol (20% in saline), centrifugation (13,000 g, 20 min), and measurement of supernatants using the RIA kit as described above. Plasma ACTH levels were measured via RIA, as described previously (44), with an intra-assay CV of 7.6% and an interassay CV of 13.3%. Plasma renin activity was determined using a commercially available kit (DiaSorin, Stillwater, MN). Briefly, angiotensin I was generated in plasma samples in the presence of phenylmethylsulfonyl fluoride to inhibit converting enzyme activity and proteolytic degradation. The amount of generated angiotensin I was then measured via RIA with an intra-assay CV of 8% and an interassay CV of 6.7%. Plasma aldosterone was assessed by RIA kit (DPC, Los Angeles, CA) with an intra-assay CV of 5.4%, an interassay CV of 14.4%, and a minimum detection limit of 25 pg/ml. When sufficient samples remained, plasma osmolality was also measured (Advanced Microosmometer; Advanced Instruments, Norwood, MA).

Adrenal RNA and DNA content. Adrenal RNA and DNA content were measured as described previously (27). In brief, adrenals were homogenized in guanidine buffer, and DNA was determined by PicoGreen assay (Molecular Probes, Eugene, OR). RNA was extracted from guanidine and quantified by absorbance at 260 nm.

Immunofluorescence histochemistry in the adrenal cortex. Immunohistofluorescence for the nuclear marker Ki67 was used to define dividing cells; previous work (27) demonstrates that Ki67 labeling during adrenal growth is similar to other markers of cell division, including proliferating cell nuclear antigen and bromodeoxyuridine. For assessment of cortical hyperplasia, staining for Ki67, P450 aldosterone synthase (P450aldo; a marker of the zona glomerulosa), and P450 11β-hydroxylase (P45011β; a marker of the zona fasciculata) were performed using a triple-labeling procedure, as described previously (27). Frozen adrenals were sectioned (30 μm), fixed in Zamboni’s solution, and incubated overnight with a mouse anti-Ki67 primary (1:50), followed by donkey anti-mouse Cy3 secondary and a blocking antibody [F(ab’2) donkey anti-mouse]. Primary antibodies directed against P450aldo (1:100; rabbit host) and P45011β (1:125; mouse host), generously supplied by C. Gomez-Sanchez, University of Mississippi Medical Center, were then applied. After overnight incubation, sections were incubated with secondary antibodies (goat anti-mouse Alexa 488 and donkey anti-rabbit Cy5) for 1 h, rinsed, and coverslipped in aqueous mounting medium (Vectashield).

For assessment of cortical hyperplasia, optical images were collected, pseudocolored, and overlapped using Adobe Photoshop. Cell counting was performed using unbiased stereology as described by Howard and Reed (40). Cells labeled for Ki67 and P450aldo or for Ki67 and P45011β were counted as proliferating glomerulosa cells or fasciculata cells, respectively. Outer fasciculata cells were classified as P45011β-positive cells adjacent to the zona intermedia; the zona intermedia expresses neither P450aldo nor P45011β. Inner fasciculata cells were classified as P45011β-positive cells adjacent to the medulla. Sampling areas within each zone were randomly selected. Proper care was taken to ensure that these sampling areas were separate and distinct regions of the fasciculata. Labeled cells within an area circumscribed by a two-dimensional box (e.g., 40.1 × 400.5 μm and 133.5 × 133.5 μm for glomerulosa and fasciculata, respectively) were counted throughout the depth of the section. Using these templates, a sampling area in the glomerulosa extended inward from the adrenal capsule and included 5–10 cell layers or ~1,000 cells; a sampling area in the fasciculata included 15–20 cell layers or ~750 cells extending inward from the zona intermedia (outer fasciculata) or
extending outward from the medulla (inner fasciculata). A total of ~200 Ki67-labeled cells were counted from 12 distinct sampling areas (2 areas from 6 different sections) for each adrenal, and the numerical density (labeled cells per volume) was determined.

To assess cellular hypertrophy in the adrenal cortex, other sections were triple labeled for Ki67, P450aldo, and P45011β, as described above, and coverslipped with aqueous medium containing a nuclear stain [4,6-diamidino-2-phenylindole (DAPI), Vectashield; Vector Labs]. Cell nuclei in the zona glomerulosa, outer zona fasciculata, and inner zona fasciculata were counted in defined areas, as described above. An increase in nuclear density was interpreted as evidence of decreased cell size, whereas a decrease in nuclear density was interpreted as evidence of increased cell size (10, 13, 38). A total of ~250 DAPI-labeled cell nuclei were counted from 18 distinct sampling areas (3 areas from 6 different sections) for each adrenal at ×40 magnification, and numerical density (labeled cells per volume) was determined.

**Immunofluorescence histochemistry in the adrenal medulla.** To assess cellular hyperplasia and hypertrophy in the adrenal medulla, other sections were single labeled for Ki67 and coverslipped with aqueous medium containing the nuclear stain DAPI, as described above. The low number of Ki67-positive nuclei observed in the adrenal medulla precluded accurate quantification in this area. To assess cellular hypertrophy in the adrenal medulla, a total of ~1200 DAPI-labeled cell nuclei were counted from 5 to 12 distinct sampling areas (1–3 areas from ~4 different sections, depending on the size of the medulla in each section) for each adrenal at ×20 magnification, and numerical density was determined. In all cases, care was taken to avoid sampling regions of the medulla that contained large blood vessels, because this would have greatly affected cellular density measurements.

**Statistics.** All data are shown as means ± SE. Statistical analyses were performed using GB-Stat, unless otherwise noted. The ability of CVS to diminish percentage of body weight gain, increase adrenal weight, and decrease thymus weight was determined by one-tailed t-test comparisons to the control group. For experiment 1, statistical differences for plasma corticosterone, adrenal corticosterone, plasma ACTH, and plasma aldosterone responses to exogenous ACTH were each compared between CVS and controls by ANOVA. Also, dose-response curves were generated and compared using GraphPad Prism. The ED50 value represents the dose of ACTH that elicits a half-maximal response, whereas the Bmax value represents the maximal response to ACTH. For experiment 2, differences in plasma ACTH, corticosterone, renin activity, and osmolality were assessed by two-tailed t-tests. Similarly, differences in adrenal RNA and DNA content were determined by two-tailed t-tests. For the adrenal cortex, the density of Ki67-positive and DAPI-positive cell nuclei were each assessed by repeated-measures ANOVA, with adrenal zone as the repeated factor. For the adrenal medulla, a difference in DAPI-positive cell densities was determined by two-tailed t-test. Fisher’s was used for post hoc analysis. Statistical significance was taken as P < 0.05.

**Table 1. CVS increased adrenal weight and decreased body weight gain and thymus weight in experiment 1**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain, %</td>
<td>20.7 ± 0.5</td>
<td>9.6 ± 0.5*</td>
</tr>
<tr>
<td>Adrenal weight, mg</td>
<td>49.1 ± 0.8</td>
<td>51.6 ± 0.9*</td>
</tr>
<tr>
<td>Adrenal weight, mg/100 g body wt</td>
<td>14.2 ± 0.3</td>
<td>16.8 ± 0.3*</td>
</tr>
<tr>
<td>Thymus weight, mg</td>
<td>464 ± 12</td>
<td>415 ± 8*</td>
</tr>
<tr>
<td>Thymus weight, mg/100 g body wt</td>
<td>134 ± 3</td>
<td>135 ± 3</td>
</tr>
</tbody>
</table>

Data are shown as means ± SE; n = 36/group. CVS, chronic variable stress. *P < 0.05 vs. control.

![Graph 1](http://ajpendo.physiology.org.org/diagram)

**Fig. 1.** Chronic variable stress (CVS) increased adrenal cortical responses to ACTH. Plasma (A) and adrenal (B) corticosterone responses to exogenous ACTH in dexamethasone-blocked rats were increased in rats with a history of CVS (■) compared with nonstress controls (○). Data are shown as means ± SE; n = 4–6/group. *P < 0.05 vs. control.

**RESULTS**

**Experiment 1.** As expected, general indexes of chronic stress were affected by the CVS procedure (Table 1). More specifically, CVS reduced body weight gain (P < 0.05), increased adrenal weight (P < 0.05), and decreased thymus weight (P < 0.05), confirming the effectiveness of the chronic stress paradigm in this experiment.

Plasma corticosterone responses to exogenous ACTH in dexamethasone-blocked rats were increased by CVS (Fig. 1A). Comparison by ANOVA revealed a main effect of stress (P < 0.05), a main effect of dose (P < 0.05), and an interaction (P < 0.05). Fitting of dose-response curves revealed that the CVS and control curves were dissimilar (P < 0.05). More specifically, the Bmax differed between the curves (control = 623 ± 54, CVS = 951 ± 89, P < 0.05), whereas the ED50 was comparable (control = 168 ± 39, CVS = 157 ± 37, P > 0.05). Plasma corticosterone levels in the absence of ACTH treatment (0 ng ACTH dose) were low and not affected by CVS (control = 6.8 ± 0.3 ng/ml, CVS = 7.1 ± 0.5 ng/ml, P > 0.05).
Table 2. Plasma ACTH after administration of exogenous ACTH to dexamethasone-blocked rats was not affected by CVS

<table>
<thead>
<tr>
<th>ACTH, ng/100 g body wt</th>
<th>Control</th>
<th>CVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>64±31</td>
<td>52±37</td>
</tr>
<tr>
<td>21.6</td>
<td>81±11</td>
<td>113±22</td>
</tr>
<tr>
<td>43.2</td>
<td>198±29</td>
<td>317±57</td>
</tr>
<tr>
<td>64.8</td>
<td>360±39</td>
<td>294±36</td>
</tr>
<tr>
<td>86.5</td>
<td>230±44</td>
<td>350±38</td>
</tr>
<tr>
<td>864.6</td>
<td>789±155</td>
<td>650±246</td>
</tr>
</tbody>
</table>

Data are shown as means ± SE in pg/ml; n = 6/group. There was a main effect of dose (P < 0.05), no effect of stress treatment (P = 0.86), and no interaction (P = 0.66).

As for plasma corticosterone, the adrenal corticosterone (ng/adrenal) response to exogenous ACTH in dexamethasone-blocked rats was also increased by CVS (Fig. 1B). Comparison with ANOVA revealed a main effect of stress (P < 0.05), a main effect of dose (P < 0.05), and no interaction (P > 0.05). CVS and control dose-response curves were different (P < 0.05), due primarily to a difference in B_{max} (control = 1,076 ± 85, CVS = 1,419 ± 217, P < 0.05) and not ED_{50} (control = 473 ± 84, CVS = 529 ± 184, P > 0.05). Adrenal corticosterone content in the absence of ACTH treatment (0 ng dose of ACTH) was low and not affected by CVS (control = 5.7 ± 0.9 ng/adrenal, CVS = 4.6 ± 1.1 ng/adrenal, P > 0.05). Moreover, the response of adrenal corticosterone normalized to adrenal weight (ng/mg adrenal; data not shown) was affected by dose of ACTH (P < 0.05), but not by CVS (P > 0.05) or by CVS-dose interaction (P > 0.05), suggesting that the CVS-induced increase in total adrenal corticosterone content was proportional to the CVS-induced increase in adrenal mass.

Plasma ACTH levels at 15 min after administration of exogenous ACTH revealed two important findings (Table 2). First, plasma ACTH was low in dexamethasone-treated rats given vehicle alone (0 ng dose of ACTH), establishing the effectiveness of the blockade of endogenous ACTH release. Second, CVS did not affect plasma ACTH levels. Although there was a dose-dependent increase (P < 0.05), there was no effect of stress (P > 0.05) and no interaction (P > 0.05). Dose-response curve fitting revealed no differences between the control and CVS curves (P > 0.05), suggesting that CVS did not affect the absorption and distribution of administered ACTH.

Plasma aldosterone levels (Table 3) after treatment with vehicle (0 ng) or low doses (21.6 and 43.2 ng) of exogenous ACTH in dexamethasone-blocked rats were below the minimum detection of the RIA (<25 pg/ml). Treatment with higher doses of ACTH increased plasma aldosterone levels (P < 0.05); however, there was no effect of CVS (P > 0.05) and no interaction (P > 0.05).

Table 3. Plasma aldosterone after administration of exogenous ACTH to dexamethasone-blocked rats was not affected by CVS

<table>
<thead>
<tr>
<th>Dose of ACTH, ng/100 g body wt</th>
<th>Control</th>
<th>CVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>21.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>43.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>64.8</td>
<td>46 ± 5</td>
<td>47 ± 8</td>
</tr>
<tr>
<td>86.5</td>
<td>60 ± 14</td>
<td>86 ± 27</td>
</tr>
<tr>
<td>864.6</td>
<td>317 ± 45</td>
<td>360 ± 52</td>
</tr>
</tbody>
</table>

Data are shown as means ± SE in pg/ml; n = 6/group; ND, not detectable (>50% of the samples were <25 pg/ml, the detection limit of the radioimmunoassay). There is a main effect of dose (P < 0.05), no effect of stress treatment (P = 0.38), and no interaction (P = 0.90) for aldosterone.

Table 4. CVS increased adrenal weight and decreased body weight gain and thymus weight in experiment 2

<table>
<thead>
<tr>
<th>Control</th>
<th>CVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain, %</td>
<td>11.1±0.6</td>
</tr>
<tr>
<td>Adrenal weight, mg</td>
<td>47.2±1.2</td>
</tr>
<tr>
<td>Thymus weight, mg</td>
<td>365±24</td>
</tr>
</tbody>
</table>

Data are shown as means ± SE; n = 6/group. *P < 0.05 vs. control.

Table 5. CVS did not affect indexes of basal HPA axis and renin-angiotensin system activity in experiment 1

<table>
<thead>
<tr>
<th>Control</th>
<th>CVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ACTH, pg/ml</td>
<td>37±10</td>
</tr>
<tr>
<td>Plasma corticosterone, ng/ml</td>
<td>56±18</td>
</tr>
<tr>
<td>Plasma renin activity, ng/ml·1·h⁻¹</td>
<td>3.0±0.5</td>
</tr>
<tr>
<td>Plasma aldosterone, pg/ml</td>
<td>ND</td>
</tr>
<tr>
<td>Plasma osmolality, mOsm</td>
<td>301±4</td>
</tr>
</tbody>
</table>

Data are shown as means ± SE; n = 6/group. (>50% of the samples were <25 pg/ml, the detection limit of the radioimmunoassay). HPA, hypothalamic pituitary adrenocortical.
fasciculata. Moreover, after CVS there was an increase in Ki67-positive cell nuclei that was restricted to the outer zona fasciculata. A few Ki67-positive cell nuclei were observed in the adrenal medulla of control and CVS rats; the incidence of these cells did not appear to be affected by CVS exposure and was too infrequent to accurately assess cellular density in this region.

Density of nuclear labeling with DAPI was assessed to estimate changes in adrenal cellular size. In the adrenal cortex (Fig. 5A), the nuclear density showed no main effect of CVS ($P > 0.05$), a main effect of cortical subregion ($P < 0.05$), and an interaction ($P < 0.05$). More specifically, in control rats nuclear density was greater in the zona glomerulosa and inner zona fasciculata than in the outer zona fasciculata. In addition, CVS increased nuclear density in the zona glomerulosa, indicative of decreased cell size in the region, whereas CVS decreased nuclear density in the inner zona fasciculata, indicative of increased cell size in this region. In the adrenal medulla (Fig. 5B), CVS decreased nuclear density, which suggests increased cell size in this region.

**DISCUSSION**

As expected, the CVS paradigm effectively produced a number of chronic stress-related changes, including increased adrenal weight, decreased thymus weight, and reduced body weight gain, as observed previously with this paradigm (36, 67) as well as with other chronic stress protocols (12, 31, 41, 49, 63). CVS enhanced the plasma corticosterone response to exogenous ACTH in dexamethasone-blocked rats specifically by augmenting the maximal response ($B_{\text{max}}$) without affecting sensitivity to ACTH ($ED_{50}$). The adrenal corticosterone content also showed a CVS-induced increase in maximal response without affecting sensitivity to ACTH, suggesting that CVS-induced alterations in plasma corticosterone are due, at least in part, to changes in adrenal corticosterone production. Importantly, plasma ACTH levels following exogenous ACTH administration were not affected by CVS. The CVS-induced enhancement of maximal corticosterone responses was associated with an increase in total adrenal DNA, RNA, and RNA/DNA ratio, suggesting that both hyperplasia and hypertrophy occurred. Cell counting of Ki67-positive cell nuclei in specific cortical zones (as defined by colabeling with zone-specific markers) demonstrated that CVS-induced hyperplasia was restricted to the outer zona fasciculata. CVS decreased the density of cell nuclei in the inner zona fasciculata and medulla, suggesting that these regions experienced cellular hypertrophy. Interestingly, CVS also increased cell nuclei density in the zona glomerulosa, suggesting that cells in this region decreased...
in size. However, indexes of renin-angiotensin system function were not affected by CVS. The present work supports the initial hypothesis that maximal adrenal responses to ACTH are augmented after chronic stress and that this increased responsiveness is associated with cellular hypertrophy and hyperplasia in the zona fasciculata. However, the data do not support the initial hypothesis of increased sensitivity to ACTH. Moreover, the results demonstrate that zona fasciculata hyperplasia occurs specifically in the outer portion of this zone; zona fasciculata hypertrophy occurs specifically in the inner portion of this zone, and hypertrophy also occurs in the adrenal medulla.

Dissociations between plasma ACTH and corticosterone are frequently observed after many types of chronic stress (8, 58, 59, 66, 84) and suggest that chronic stress increases adrenocortical responsiveness, particularly in the morning near the nadir of the circadian rhythm (59). In vitro work (1) using

Fig. 3. Adrenal sections collected from control (A) and CVS rats (B) and immunolabeled for P450 aldosterone synthase (P450aldo; blue), P450 11β-hydroxylase (P45011β; green), and Ki67 (red). ZG, zona glomerulosa; ZI, zona intermedia; ZF, zona fasciculata. Examples of the 2-dimensional boxes used for unbiased stereology are shown for the ZG (white rectangle; 40 × 200 μm; note that the rectangle depicted is ½ the length of the rectangle that was actually used) and the ZF (white square; 133 × 133 μm). Scale bar = 100 μm.

Fig. 4. CVS induced cellular hyperplasia selectively in the outer zona fasciculata. Density of Ki67-positive cells in adrenals from CVS (closed bars) vs. control rats (open bars) was increased in the outer ZF (oZF) but not in the ZG or the inner ZF (iZF). Cellular zones were defined by colabeling with antisera directed against P450aldo and P45011β, as described in MATERIALS AND METHODS; n = 6/group. *P < 0.05 vs. control; #P < 0.05 vs. oZF.

Fig. 5. CVS induced cellular hypertrophy in the inner ZF and medulla, whereas CVS decreased cell size in the ZG. Density of 4,6-diamidino-2-phenylindole (DAPI)-positive cells in the cortex (A) and medulla (B) of adrenal glands from CVS (closed bars) and control rats (open bars); n = 6/group. *P < 0.05 vs. control; #P < 0.05 vs. oZF.
several ACTH doses in dispersed zona fasciculata cells demonstrates that ACTH evokes greater maximal cAMP, pregnenolone, and corticosterone production in cells taken from rats with previous chronic stress, with no effect on the ED50. Previous in vivo tests of adrenal responses used one to two doses of ACTH, thereby precluding definitive differentiation between the effects of chronic stress on maximal adrenocortical responses vs. sensitivity to submaximal doses of ACTH (7, 8, 70, 80). Administration of a single supramaximal dose of ACTH to rats after chronic variable stress (8) or repeated restraint stress (70) results in increased plasma corticosterone levels. Moreover, after chronic immobilization stress, the plasma corticosterone response to a large dose of ACTH (100 mU) is increased, whereas the response to a lower dose of ACTH (20 mU) is not different (7). Also, plasma corticosterone responses to lower doses of ACTH (50 and 100 ng) are not affected by previous chronic cold stress (80). Collectively, these findings are consistent with the present results demonstrating increased maximal corticosterone responses to ACTH but no change in sensitivity to ACTH. It should be noted that the present study was conducted near the nadir of the circadian rhythm. It has been suggested that the effect of chronic stress on adrenocortical responsiveness varies throughout the activity cycle (59), and future studies should address whether chronic stress alters the diurnal rise in adrenal sensitivity to ACTH that occurs at the peak of the circadian rhythm (19).

In the present studies, the observed increase in maximal corticosterone responses to ACTH is associated with a proportional increase in adrenal mass. Enlargement of the adrenal cortex has been reported after several types of chronic stress (1, 33, 73, 74, 78). More specifically, in the zona fasciculata cellular hypertrophy occurs after chronic toluene exposure (34), and both hypotrophy and hyperplasia are seen during streptozotocin-induced diabetes (69). However, growth and function of the zona fasciculata may vary across the zone. For example, hyperplasia in response to acute ACTH administration, surgery, or compensatory adrenal growth occurs primarily in the outer zona fasciculata (27, 62). The present work shows that CVS, a chronic stress model that produces many depressive-like symptoms, induces hyperplasia in the outer zona fasciculata and hypertrophy in the inner zona fasciculata. These data support the idea that both cellular hypertrophy and hyperplasia in the zona fasciculata contribute to chronic stress-induced adrenal growth in a subregion-specific manner.

The effects of chronic stress on the zona glomerulosa are generally opposite from the effects on the zona fasciculata. For example, chronic hypoxia decreases the size of the zona glomerulosa and its cells (54, 83), similar to the decreased cell size observed after CVS in the present studies. Moreover, chronic stress results in diminished expression and activity of P450aldo (1, 68) and decreased maximal aldosterone responses in vitro (2). In the present studies, indexes of basal renin-angiotensin system function and ACTH-stimulated aldosterone production were not altered by CVS. However, the aforementioned in vitro work (2) suggests that CVS may affect maximal angiotensin II-stimulated aldosterone production in vivo; future work should address this interesting possibility.

The differential effects of chronic stress on the adrenocortical zones, in which cells of the zona fasciculata undergo growth (hyperplasia and hypertrophy) and cells of the zona glomerulosa decrease in size, could occur through multiple, possibly overlapping, mechanisms. For example, repeated exposure to elevated plasma ACTH during chronic stress may stimulate both zona fasciculata growth and zona glomerulosa atrophy. During prolonged treatment with ACTH there is an initial increase in adrenal weight, RNA, and protein that is later followed by an increase in adrenal DNA (3, 20, 28, 42). Prolonged ACTH treatment increases the volume of zona fasciculata cells (65) and the expression of P450 side-chain cleavage mRNA in the zona fasciculata (1, 53). Moreover, stress associated with surgery induces hyperplasia specific to the outer zona fasciculata that is prevented by dexamethasone pretreatment (27). In contrast, although acute administration of ACTH stimulates aldosterone steroidogenesis (30), prolonged ACTH administration impairs zona glomerulosa function (1, 53, 76). Collectively, this work suggests that long-term ACTH treatment induces an initial cortical hypertrophy followed by a delayed cortical hyperplasia that is largely localized to the zona fasciculata and is coupled with attenuated function of the zona glomerulosa. A primary role for ACTH is also supported by the observation that, during repeated restraint, lesions of the mediodorsal hypothalamus or PVN decrease the ACTH response to stress and prevent the chronic stress-induced increase in adrenal weight (84). Besides ACTH, pituitary-derived NH2-terminal proopiomelanocortin peptides and the adrenal innervation have been implicated in mediating adrenal growth (21, 55, 79) and may also be involved in regulating the effects of chronic stress.

Chronic stress also has effects on the adrenal medulla, as reflected by the present demonstration of cellular hypertrophy, but not hyperplasia, in this region. Increased adrenal medullary size and/or catecholamine content are frequently observed after other types of chronic stress (33, 60, 71, 73, 83). Moreover, after chronic stress there is a generalized increase in medullary function (11, 17, 26, 50, 51, 60, 61), suggesting that medullary hypertrophy may be a general consequence of chronic stress. Chronic stress-induced hypertrophy of the adrenal medulla likely results, at least in part, from repeated activation by the sympathetic nervous system (60). However, ACTH and glucocorticoids can also affect medullary catecholamine content, enzyme expression, and/or enzyme activity (29, 52, 61, 75), suggesting that hormones may contribute to medullary hypertrophy after chronic stress. It should be noted that the purpose of the present studies was to characterize the effects of CVS on cortical growth and corticosterone steroidogenesis. The observed hypertrophy of the adrenal medulla is intriguing, and future work should include experiments to determine whether the hypertrophy is accompanied by alterations in medullary function.

Depression is a chronic stress-related disorder, and many patients with depression have altered HPA axis function that is generally characterized by increased HPA axis activity (14, 15, 18, 71, 72). This HPA hyperactivity is largely believed to result from increased central drive to the axis, resulting in increased CRH and ACTH release. However, it has also been suggested (4, 18) that increases in adrenal size and/or function, perhaps as a consequence of increased central HPA axis activation, may additionally contribute to hypercortisolism in depression. For instance, depressed patients and victims of suicide (a high proportion of whom suffered from depression) often have enlarged adrenal glands (4, 24, 25, 64, 77). This increase in adrenal weight is associated with increased size of the adrenal...
cortex (77, 81), and many depressed patients, particularly those resistant to dexamethasone blockade, have exaggerated cortisol responses after ACTH administration (5, 43, 45). Collectively, this work suggests that many depressed patients show increased adenocortical size and zona fasciculata function. Additionally, patients with depression have greater urinary output of epinephrine and norepinephrine indicative of increased adrenomedullary function (48, 56), suggesting that hypertrophy of the adrenal medulla may also contribute to increased adrenal weight during depression. In fact, increased medullary activity has been implicated in the impaired cardiovascular function that is observed in some depressed patients (26).

In summary, CVS increases the maximal adrenal corticosterone response to ACTH without affecting sensitivity, suggesting that corticosterone levels may be particularly elevated during times of high stimulation, such as during acute stress. This increase in cortical function is paralleled by hypertrophy of the inner zona fasciculata and medulla and hyperplasia that is limited to the outer zona fasciculata. These data support the idea that chronic stress-induced adrenal growth produces alterations in adrenal function that may have implications for patients with stress-related disorders.

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