Bilateral adrenal enucleation-induced changes in adenohypophyseal pro-opiomelanocortin (POMC)-related peptides synthesis and secretion: A comparative study with adrenalectomized rats

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ABSTRACT. The aim of the present study was to elucidate the modulatory effect of transient changes in endogenous glucocorticoids, occurring after bilateral adrenal enucleation (ENUC), on anterior pituitary (AP) proopiomelanocortin (POMC)-derived peptides synthesis and output in rats. For this purpose, adult female rats were either bilaterally ENUC, adrenalelectomized (ADX), or sham-operated (SHAM) and killed by decapitation 2, 7, 14, and 21 days after surgery. Trunk blood was collected for measurements of ACTH, β-endorphin (β-END) and corticosterone (B) concentrations; APs were quickly dissected for the determination of ACTH, β-endorphin (β-END)-like (β-END-LI) and γ_3-MSH contents and adrenal glands were removed and submitted to histological study. The results indicate that ENUC and ADX increased AP POMC-related peptides synthesis and release in association with changes in the AP processing of peptides belonging to the N-terminal (γ_3-MSH), mid (ACTH) and C-terminal (β-LPH/ENDs) portions of POMC. While ADX abolished plasma B levels, ENUC induced a transient (day 2) decrease in plasma B concentrations which returned to SHAM levels at 7 days after surgery. These data tallied with the histological observations carried out, indicating a time-dependent regenerative process of the adrenal which was completed by three weeks after ENUC. There was a different pattern in plasma ACTH and β-END levels between ENUC and ADX; maximal plasma peptide levels were found 7-14 days after ENUC, then falling down to SHAM values at 21 days post ENUC. Conversely, there was a constant increment in plasma peptide levels up to 21 days after ADX. At 2 days after both ENUC and ADX all peptides measured in the AP were lower than SHAM values, thus reflecting a rapid corticotrope secretion. Thereafter, 7 or more days after surgery, AP peptide content in ADX rats increased, in a time-related fashion, up to 21 days after surgery. Only β-END-LI showed a similar AP content to that of the SHAM group, thereafter indicating a preferential cleavage of POMC to β-END long after ADX (21 days). ENUC rats showed increased AP POMC peptides content throughout the whole time, and it was significantly different from SHAM and ADX values 14 days postsurgery. Interestingly, we found an increment in AP γ_3-MSH, a peptide which is preferentially synthesized in the intermediate lobe of the rat pituitary, in both ENUC and ADX situations. Our results further indicate that: 1) glucocorticoids, from regenerating adrenal origin, induce a fast negative feedback mechanism on AP secretion, and 2) there might be a delayed inhibitory action of newly synthesized corticosteroids on higher levels of the central nervous system. The lack of glucocorticoids (ADX) clearly corroborates a persistent enhancement of AP POMC-related peptides synthesis and secretion. The differences in AP processing of POMC between ENUC and ADX might be due to qualitative/quantitative changes in hypotalamic ACTH secretagogues output.

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INTRODUCTION

Rat adrenal regeneration is a phenomenon which occurs after either unilateral adrenalectomy combined with contralateral enucleation or bilateral adrenal enuclation (ENUC). Different hypophyseal and non-hypophyseal hormones as well as neuropeptides exert trophic effects on adrenal glands

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(1). Since hypophysectomy abolishes the adrenal regenerative process, it has been suggested that such an effect is fully dependent on intact corticotrope function (2, 3). It is assumed that the decrease in circulating levels of corticosteroids as a consequence of ENUC triggers the adrenal regenerative process; the treatment with either adrenal extract or corticosteroids in ENUC rats fully abolishes such a process (3, 4). The effect induced by corticosteroids indicates that corticotropes are the main source of substances with trophic/mitogenic action on the adrenals.

Until the eighties, it was assumed that $ACTH_{1-39}$ was the most relevant end product of corticotropine cells. A large body of evidence suggests that ACTH is the main hypophyseal hormone with a physiological role in both adrenal growth and activity. In addition to the stimulatory effect of ACTH on corticosteroid release, *in vivo* administration of ACTH increases adrenal mRNA and protein synthesis (5, 6). Also chronic treatment with ACTH induces dramatic morphological changes in the adrenal cortex (7, 8). however, it is known that corticotropes also synthesize and release a great number of peptides aside from ACTH; they belong to a common protein precursor, the so-called pro-opiomelanocortin (POMC).

The biosynthesis and postranslational processing of POMC have been extensively investigated, and it has been shown that these processes differ according to the lobe of the pituitary gland that is involved. In the rat anterior pituitary gland, POMC is firstly cleaved to $ACTH_{1-39}$, $N\text{-}POMC_{1-74}$ and β lipotrophin (9), whereas in the intermediate lobe of the rat pituitary, the process continues to peptides smaller than those produced by the anterior lobe, such as α-melanotropic hormone (α-MSH), corticotropin-like intermediate lobe peptide (CLIP), β-endorphin (β-END) and β-END-related peptides. Also the N-terminal region of POMC (N-POMC) is subjected to more extensive cleavange in the intermediate lobe, generating $N\text{-}POMC_{1-49}$ and Lys-γ_3-MSH (10, 11).

In addition to the well-known trophic effect of ACTH on the adrenal gland, it was found that other N-POMC-related peptides, such as $N\text{-}POMC_{1-48}$, are reliable physiologic mitogenic stimuli on adrenal glands (2); the source of these mitogenically active N-POMC-derived peptides seems to be the anterior pituitary lobe (2). Since the N-POMC processing in the rat anterior pituitary after bilateral adrenalectomy (ADX) and during adrenal regeneration has only been partially investigated (12-14), the aim of the present experiment was to determine whether the changes in N-POMC processing at the corticotrope level extended to other POMC regions after ENUC. For this purpose we examined, by specific assays, not only the N-terminal POMC region (γ_3-MSH) but also the mid (ACTH) and C-terminal (β-LPH/END) POMC portions in two different conditions: a) in animals with transient changes in plasma glucocorticoid levels (ENUC) and b) in glucocorticoid-depleted matched animals (ADX).

MATERIALS AND METHODS

Chemicals

All reagents used were of analytic grade. Bovine serum albumin (BSA, radioimmunoassay grade), actived charcoal, dextran (MW 70,000), Na_2EDTA, 2-mercaptoethanol, 2,5-dipheniloxazole (PPO), 1,4-Di-2-(5-pheniloxazol)-benzene (POPOP), polyethylene glycol (PEG) and potassium sodium tartrate were purchased from Sigma Chem. Co., St Louis, MO, U.S.A. $Na^{125}I$ and ^3H-Corticosterone (specific activity 88 Ci/mmol) were from New England Nuclear, Boston, MA, U.S.A. Chloramine T, sodium azide, sodium metabisulfite were purchased from Mallinckrodt Works, U.S.A. Sodium chloride, sodium hydroxide, disodium phosphate, monosodium phosphate, dichloromethan, toluene, dioxane, trifluoroacetic acid and Folin-Ciocalteu's reagent were from Carlo Erba, Milan, Italy. Hydrochloric acid, acetone, methanol and acetic acid were from Merck Laboratories Argentina. $ACTH_{1-39}$ β-endorphin and γ_3-MSH were from Peninsula Laboratories, Belmont, CA, U.S.A.

Animals and surgeries

Two-month-old, randomly cycling, female Sprague-Dawley rats were used in this study. Animals were kept at 21±1 C, on a 12 h light: 12 h darkness cycle (07:00 - 19:00 h lights on), with rat chow available *ad libitum*. ENUC and ADX as well as SHAM operations were performed by the dorsal approach under light ether anesthesia as earlier reported (15). Briefly, adrenal enucleation was carried out by exposing the adrenals and making a puncture in the capsule; the parenchyma was then extruded by squeezing the gland gently with forceps, leaving the capsule intact. SHAM-operation consisted of explosing both adrenals without touching the glands or their pedicles. ADX rats drank 0.9% (w/v) NaCl until decapitated, while ENUC also drank 0.9% (w/v) NaCl but only for the first 4 days after surgery; thereafter they drank tap water until the end of the experiment. While the success of ADX was monitored by undetectable plasma B concentrations and confirmed after autopsy of animals, adrenal glands from ENUC-rats explored by microscopical analysis.

Experimental design

Different groups of rats (8-10 animals per group) were housed in individual cages 24 h before they were killed. Animals were decapitated (between 08.30 and 09.30) on days 2, 7, 14 and 21 after surgery and trunk blood was collected in plastic tubes containing 0.5 ml of EDTA solution (10% W/V) and centrifuged for 15 min at 4 C. Plasma samples were aliquoted and stored at 20 C until the measurement of ACTH, β-END and B concentrations. Anterior pituitary (AP) lobes were quickly dissected after the removal of brain tissues, then sonicated for 15 seconds in 0.5 ml HCl (0.1 M); these homogenates were centrifuged at 13,000 x g for 3 min and the supernatants were stored frozen (-20 C) until determination of hormone and protein contents. Some APs were subjected to routine histological techniques for microscopical observation in order to ensure that they were not contamined with neurointermediate lobe tissue. Adrenal glands (AG) were removed, free of adipose tissue, immediatly after decapitation; glands were then fixed in Bouin's fluid and processed by histological techniques for microscopic studies.

Histological analysis of adrenal glands

Both adrenal glands of 4 representative rats from each group were fixed in Bouin's fluid, embedded in paraffin and sectioned serially at 5 µm. The sections were stained with hematoxylin and eosin.

Hormone assays

ACTH. Plasma and AP ACTH were determined by a two-site immunoradiometric assay (IRMA) as earlier reported (16). Briefly, 200 µl of sample or standard ($ACTH_{1-39}$, range 5-1,000 pg/ml) was diluted in 0.05 M sodium phosphate buffer, pH 7.4, 0.025% (W/V) BSA and incubated overnight at 4 C in the presence of 100 µl (^{125}I)-sheep anti-$ACTH_{1-24}$ IgG (approximately 100,000 cpm) and 100 µl of rabbit anti-$ACTH_{35-39}$ serum (1/1,000) in 0.05 M sodium phosphate buffer, pH 7.4, containing normal rabbit serum (1% V/V), BSA (0.2% W/V) PEG (2% W/V). Separation of bound from free labeled IgG was achieved by the addition of 200 µl sheep anti-rabbit IgG serum diluted 1/20 in 0.05 M sodium phosphate buffer, pH 7.4. The reaction was kept at room temperature for 1 h and then centrifuged at 4 C. The bound fractions was washed with 2 ml of PEG solution (2% W/V), Triton x 100 0.01% (V/V) in 0.05 M phosphate buffer, pH 7.4. Bound radioactivity was measured in a Kontron (Switzerland) gamma-counter. The intra- and interassay coefficients of variation were 2 and 6% respectively.

γ_3-MSH. AP Y_3-MSH was determined by radioimmunoassay (RIA), which has been detailed elsewhere (17). ^{125}I-y3-MSH was used as tracer, and was prepared by the Chloramine T method. AP homogenate or standard (γ_3-MSH; range 20-5,000 pg/ml) was diluted in 200 µl of 0.05 M sodium phosphate buffer, pH 7.4 containing 2-mercatoethanol 0.2% (V/V), 0.25% (W/V) BSA, and incubated overnight at 4 C with 50 µl γ_3-MSH antiserum, coded MC7 and developed in rabbit, at a dilution that binds 30% of tracer in the absence of standard. MC7 antiserum is specific for γ_3-MSH and Lys-γ_3-MSH and had no cross-reactivity with N-$POMC_{1-48}$ or POMC and less than 0.1% with N-$POMC_{1-77}$. The reaction was stopped by the addition of 200 µl 0.25% (W/W) dextran-1% (W/W) charcoal mixture in 30% (V/V) horse serum diluted in 0.9% (W/V) sodium chloride solution. After 20 min incubation at 0 C, tubes were centrifuged for 20 min at 4 C. Supernatants were discarded and free radioactivity in the charcoal-dextran pellet was counted. The intra- and interassay coefficients of variation were 6 and 12% respectively.

β-endorphin-like (β-END-Li). AP β-end-Li was determined by RIA, and the antiserum used was kindly provided by the National Hormone and Pituitary Program (USA). The antiserum (50 µl), developed against human β-Lipoprotein, possesses full cross-reactivity with rat β-endorphin and related peptides. Rat ^{125}I-β-endorphin was used as tracer (9,000-10,000 cpm per 50 µl). The assay's buffer consisted in 0.25% (W/V) BSA, 0.2% (V/V) mercaptoethanol in 0.05 M sodium phosphate buffer, pH 7.4. Tracer and standard or sample, diluted in 200 µl assay buffer, were added into plastic tubes. The incubation was stopped by addition of 200 µl of 0.25% (W/V) dextran-1% (W/V) charcoal suspended in 30% (V/V) horse serum in 0.9% (W/V) sodium chloride solution. After 20 min incubation at 0 C, tubes were centrifuged for 20 min at 4 C and decanted, then free radiocativity was counted. The intra- and interassay coefficients of variation were 7 and 13%, respectively.

β-endorphin. Plasma and AP β-END were determined by a two-site, highly sensitive, IRMA purchased to Nichols Diagnostics Institute (San Juan Capistrano, CA, USA). This method has been validated for rodents in our laboratory. The intra- and interassay coefficients of variation ranged between 2-4 and 6-8%, respectively.

Corticosterone. Plasma (dichloromethan-extracted) B concentrations were determined as previously reported by using a specific anti-B rabbit serum (18) The anti-B rabbit serum had been developed in our laboratory (18), it has a cross-reactivity of: 13,5% with dexamethasone, 2.2% with compound

F, 1.1% with compound S, 1% with progesterone, 0.45% with 17-alpha-hydroxy-progesterone, 0.23% with testosterone, 0.13% with 4-adrosterone-3, 17-dione, 0.08% with aldosterone and less than 0.003% with estradiol, estriol and compound E. The intra- and interassay coefficients of variation ranged between 4-7 and 8-10 respectively.

Statistics

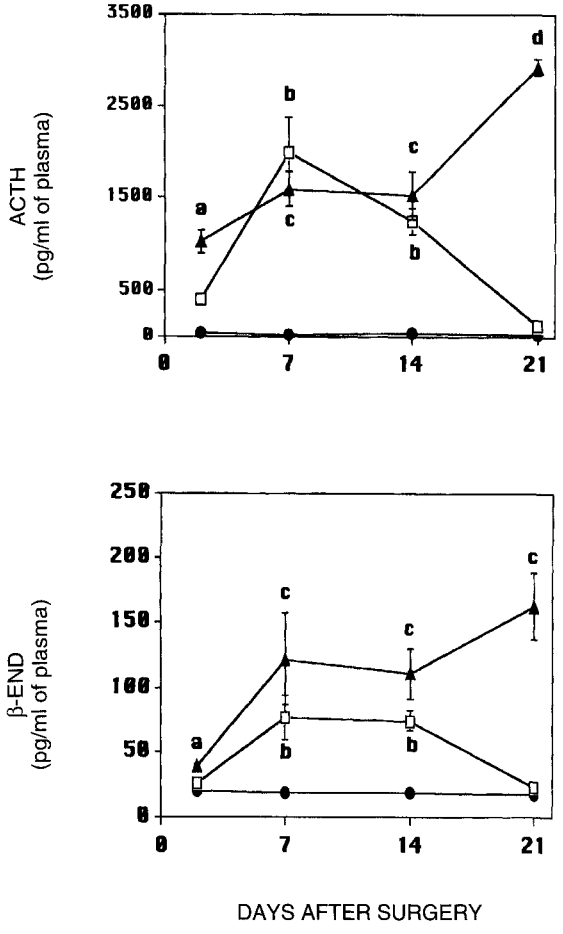
Data were analyzed by analysis of variance (multifactorial) followed by Fisher's test for comparison of different mean values (19).

RESULTS

Time-course of ACTH, β-END and B plasma levels after bilateral ENUC and ADX

As seen in Figure 1 (upper panel), plasma ACTH concentrations remained low (at the "non-stress level": values of the same order of magnitude as those obtained in intact animals killed in similar conditions, data not shown) in SHAM animals throught the entire experiment. Plasma ACTH levels at different periods after either ENUC or ADX are also included in Figure 1 (upper panel). Two days after surgery, plasma ACTH levels were significantly ($p<0.01$) increased in ENUC and ADX group over SHAM values. However, in this period following surgery, plasma ACTH levels in ADX animals were significantly ($p<0.01$) higher than those found in ENUC rats. In enucleated animals, plasma ACTH levels were maximal on day 7 after ENUC ($p<0.001$ versus SHAM), them declined on day 14 after ENUC (but remaining significantly higher than SHAM values; $p<0.001$), and were finally similar to SHAM values on day 21 after ENUC. In contrast, circulating ACTH concentrations in ADX rats were several-fold higher ($p<0.001$) than SHAM levels on days 7 and 14 post surgery; they reached a maximal mean value 21 days after ADX ($p<0.001$ *vs.* SHAM).

The pattern of plasma β-END levels at different periods after surgery in the three groups of rats is shown in Figure 1 (middle panel), a patten essen-



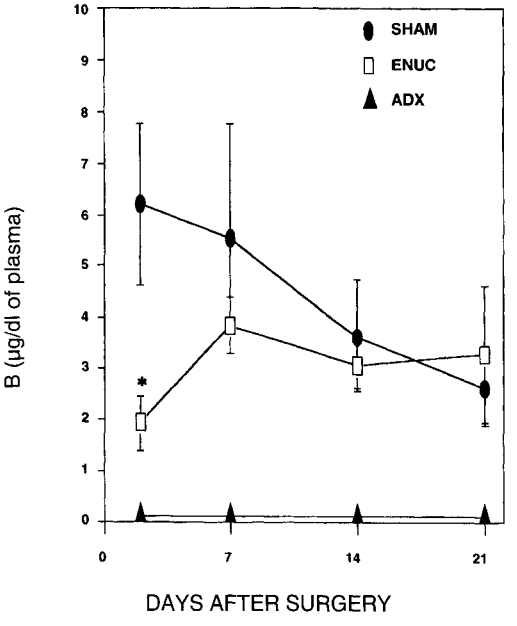


Fig. 1 - *Circulating levels of ACTH (upper), β-END (middle) and corticosterone (lower) in adrenalectomized- (ADX), enucleated- (ENUC) and SHAM-operated rats on different days after surgery. Points represent the mean(±SE) of 8 rats per group. All points except 2 day (β-END) and 21-day (ACTH and β-END) ENUC values, were significantly (p<0.05 or less) higher than the respective SHAM-value. B values in ADX rats were not detectable. a, p<0.05 or less vs. ENUC-2 values; b, p<0.05 or less vs. ENUC-2 and ENUC-21 values; c, p<0.05 vs. ADX-2 values; d, p<0.05 vs. ADX-2, ADX-7 and ADX-14 values; *, p<0.05 vs. SHAM-2 values.*

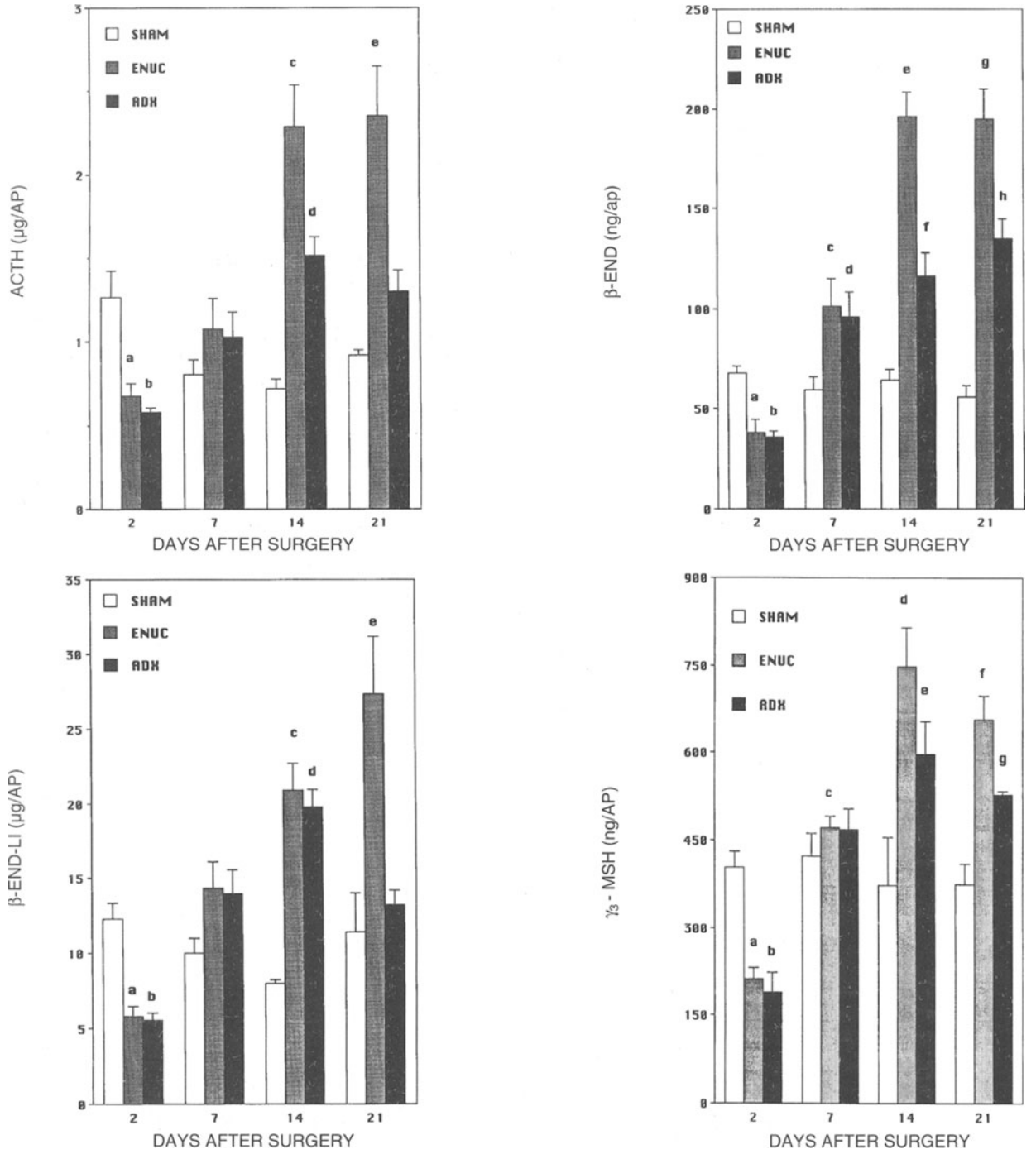


Fig. 2 - *Time-course of POMC-related peptides [ACTH (upper left panel), β-END (upper right panel), β-END-LI (lower left panel and γ3-MSH (lower right panel)] contents in rat AP on different days after surgery. Bars represent the mean (±SE) of 8 rats per group. a,* $p<0.05$ *or less* vs. *SHAM-2, ENUC-14 and ENUC-21 values; b,* $p<0.05$ *or less* vs. *SHAM-2, ADX-7, ADX-14 and ADX-21 values; c,* $p<0.05$ *or less* vs. *SHAM-14, ENUC-7 and ADX-14 values; d,* $p<0.05$ *or less* vs *SHAM-14 and ADX-7 values; e,* $p<0.01$ *or less* vs. *SHAM-21, ADX-21 values; f,* $p<0.01$ *or less* vs. *SHAM-2, ENUC-7, ENUC-14 and ENUC-21 values; g,* $p<0.05$ *or less* vs. *SHAM-7, ENUC-14 and ENUC-21 values; h,* $p<0.05$ *or less SHAM-7 values; i,* $p<0.01$ *or less* vs. *SHAM-14 and ADX-14 values; j,* $p<0.01$ vs. *SHAM-14 values; k,* $p<0.05$ *or less* vs. *SHAM-21 values; l,* $p<0.05$ *or less* vs. *SHAM-14, ADX-7 and ADX-21 values; m,* $p<0.01$ vs. *SHAM-21, ENUC-7 and ADX-21 values; n,* $p<0.001$ vs. *ENUC-14 and ENUC-21 values; o,* $p<0.05$ vs. *SHAM-14 and ENUC-7 values.*

tially similar to that of ACTH was found. Plasma β-END levels were also low and within the "non-stress" range during the entire experiment in SHAM animals. Plasma β-END levels increased significantly ($p<0.001$) over SHAM and ENUC values ($p<0.01$) on day 2 after ADX. On days 7 and 14 after either ENUC or ADX, plasma β-END levels were significantly ($p<0.01$) higher than the respective SHAM values. Then, β-END levels in ENUC rats declined to SHAM values by day 21 after surgery, whereas on day 21 after ADX, plasma β-END levels were still several fold higher ($p<0.0001$) than SHAM values.

Finally (Figure 1, lower panel), we found that plasma B concentrations in SHAM animals were also within the "non-stress" range during the entire experiment. As expected, 2 days after ENUC we found a significant decrease ($p<0.05$), in comparison to day-2 SHAM value, in plasma B levels. However, ENUC rats recovered plasma B concentrations similar to SHAM levels on day 7 after operation; this parameter remained unchanged until the end of the experiment. Figure 2 also shown that ADX animals had plasma B levels on the low detection limit of the assay, regardless of the experimental day.

Changes in APPOMC-related peptides after ENUC and ADX

The results of AP ACTH content in rats killed 2, 7, 14, and 21 days after surgery (SHAM, ENUC and ADX) are shown in Figure 2 (upper left panel). On day 2 after surgery, AP ACTH content in animals from both ENUC and ADX groups decreased significantly ($p<0.01$) *vs.* SHAM values. Thereafter, AP ACTH recovered SHAM values in both experimental groups (ENUC and ADX) on day 7 after surgery. Then, this parameter was significantly ($p<0.01$) higher than SHAM levels on day 14 after both ADX and ENUC; however, AP ACTH in ENUC animals was significantly ($p<0.01$) higher than in ADX rats. Similarly to the patten found on day 14, AP ACTH values in 21-day ENUC rats were significantly ($p<0.01$) greater than those found in both SHAM and ADX animals, although on this day AP ACTH in ADX rats was not significantly different from SHAM values.

Similarly to AP ACTH, AP β-END (Figure 2, upper right panel) was significantly ($p<0.01$) lower than SHAM values on day 2 after both ENUC and ADX; 7 days post-surgery, AP β-END was significantly ($p<0.05$) greater than the respective SHAM values in both experimental groups (ENUC and ADX). On days 14 and 21 after surgery, while AP β-END remained similar to 7-day values in both SHAM and ADX groups, it was significantly increased in 14-day ENUC over SHAM ($p<0.01$) and ADX ($p<0.01$) as well as over ENUC values on day 7 ($p<0.0001$). AP β-END-LI content (Figure 2, lower left panel), on days 2 and 7 after surgery was almost similar to that observed for both AP ACTH and β-END. On day 14 after treatment there was a significant ($p<0.01$) increment in AP β-END-LI in both ENUC and ADX groups over SHAM values; at 21 days after surgery, while ADX values were similar to SHAM values, ENUC values were significantly ($p<0.01$) higher than both SHAM and ADX.

Finally, changes in the N-terminal region of POMC were determinated by measuring AP γ_3-MSH content at different periods after surgery. The patten of this peptide throughout the experiment was similar to those described above (Figure 2, lower right panel). A significant ($p<0.01$) decrease in AP γ_3-MSH occurred 2 days after both ENUC and ADX compared to the respective SHAM value. On day 7 after both type of surgery, both experimental groups recovered SHAM values. On experimental day 14, both groups (ENUC and ADX) showed a significant ($p<0.05$ or less) increase over 14-day SHAM values; thereafter, this pattern continued to day 21 after surgery, although AP γ_3-MSH in ENUC rats was significantly ($p<0.05$) higher than in ADX animals.

Histological appearance of regenerating adrenal glands

In SHAM-operated animals (Fig. 3A) the histological appearance of the adrenal gland present the characteristics of the normal organ.

Two days after ENUC (Fig. 3B), the capsule was edematous, was thicker than normal, and contained distended blood vessels. Some cells were swollen and vacuolated. Strands of cortical cells extended down from the capsule all around the section. The staining of these regenerating cells varied in intensity. Occasional mitotic figures were found in parenchymal cells. The central area was filled with a blood clot containing cellular debris, polymorphonuclear leukocytes and hemosiderin. Connective tissue cells appeared between the inner zone of the regenerating cortex and the clot.

Seven days after ENUC (Fig. 3C), the capsule was thinner and less edematous. Cortical tissue extended further inward from the capsule, mainly in parallel columns which sometimes appeared distorted. A moderate number of mitoses could be observed in the parenchymal tissue. Numerous fibroblasts were seen beneath the cortical cells. In the central zone, the clot was being reabsorbed and replaced by scar tissue. Dilated blood vessels could be found in this area.

Fourteen days after ENUC (Fig. 3D), the capsule was approximately normal in structure and thickness. The

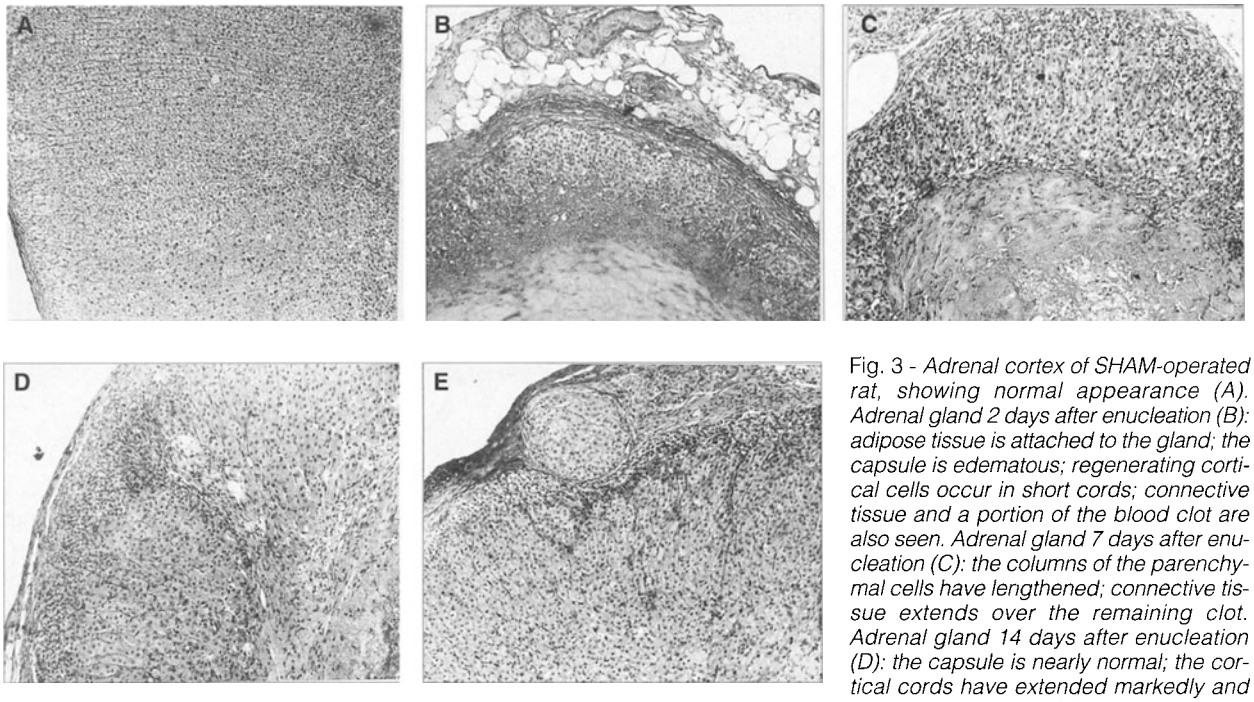


Fig. 3 - *Adrenal cortex of SHAM-operated rat, showing normal appearance (A). Adrenal gland 2 days after enucleation (B): adipose tissue is attached to the gland; the capsule is edematous; regenerating cortical cells occur in short cords; connective tissue and a portion of the blood clot are also seen. Adrenal gland 7 days after enucleation (C): the columns of the parenchymal cells have lengthened; connective tissue extends over the remaining clot. Adrenal gland 14 days after enucleation (D): the capsule is nearly normal; the cortical cords have extended markedly and appear distorted in some places; connective tissue is more compact. Adrenal gland 21 days after enucleation (E): the three normal layers of the cortex have developed completely; areas of parenchymal cells arranged in whorls (upper left corner) are frequently found. (Obj. x 10)*

new adrenal cortex had nearly reached its normal extension, and both the zona glomerulosa and the zona fasciculata were well defined. In some areas, the cell columns were arranged in whorls. Mitotic figures were also observed in this period. The central zone exhibited compact fibrous tissue containing macrophages with blood pigments.

Finally, twenty-one days after ENUC (Fig. 3E), the three normal layers of the adrenal cortex were completely developed. However, strands of connective tissue frequently appeared extending from the capsule and separating areas of cortical cells arranged in several major whorls. Remaining scar tissue containing dilated vessels and macrophages with blood pigments were found in the central zone.

It is important to point out that adrenal medullary cells did not remain in adrenal glands from different groups of ENUC rats (Fig. 3, A-E).

DISCUSSION

The present study demonstrates that AP POMC processing varied throughout the time after bilateral adrenal enucleation and adrenalectomy, and that this processing is different between ENUC and ADX rats. Moreover, we have shown time-dependent changes in AP POMC-related peptides content during adrenal regeneration, which correlate well the biochemical and morphological adrenal responses.

As earlier reported (14), we found shortly after ENUC (day 2) a decreased circulating level of adrenal glucocorticoid which coincided with high plasma ACTH concentration. At this period of time, we found an enhanced amount of glucocorticoid in the regenerating adrenal (data not shown); this fact could be dependent on increased ACTH input by the adrenal, combined with a diminished negative feedback mechanism of peripheral glucocorticoids at the pituitary level. There could also be a decrease in adrenal glucocorticoid output at this time, since the vascularization of the gland could be not completed as a consequence of tissue damage. On day 7 after ENUC, similarly to that reported at 10 days in this model (15) adrenal glucocorticoid content and secretion were normalized at the same time that dilated blood vessels appeared in the regenerating adrenal gland; it could be assumed that the increase of both synthesis and release of adrenal glucocorticoid occurred as a consequence of a still enhanced plasma ACTH level. These observations tally wiyh an early report of Holzwarth et al. (15) and clearly support an impaired sensitivity of the regenerating adrenals to higher circulating ACTH levels in terms of glucocorticoid secretion thereafter, four-

teen days after ENUC, when plasma ACTH levels started to decrease, the new adrenal cortex was nearly normal in extension with well defined zona glomerulosa and fasciculata; it then appeared as a normal gland 21 days after ENUC when plasma ACTH levels were back to the basal range.

It has been demonstrated that different factors such as vasopressin (20), angiotensin II (21) and insulin-like growth factor (22) exert trophic activities upon the adrenal cortex. At the present time, it is known that this regenerative process is fully dependent on AP function, since it has been reported that hypophysectomy abolishes such an effect (2, 3). It has been demonstrated that small N-POMC-related peptides, of corticotrope origin, have mitogenic activity upon the adrenal gland (2); in fact, these peptides derive from the N-terminal region of processed POMC after ENUC in rats (12). Presently, we determined some changes in AP N-POMC processing after ENUC, by the examination of different regions of the hormone precursor, such as γ_3-MSH (N-terminal), ACTH (middle region) and β-LPH/END (C-terminal). Changes in POMC processing after ENUC (12) have been attributed to an effect due to changes in the production of adrenal corticosteroids. Holzbauer et al. (23) found that, although no differences between SHAM and 4-day-enucleated rats in deoxycorticosterone production were found, ENUC animals did produce lower amount of 18-hydroxy-deoxycorticosterone than SHAM animals did. For that reason, the use of ADX rats is important in order to compare corticotrope function in the two conditions: ENUC and ADX. In our experimental design, several POMC-related peptides decreased in the AP 2 days after surgery (ENUC and ADX) in comparison the SHAM values. The absence of circulating glucocorticoids may explain the low AP peptide content, such as ACTH, immediately after ADX (24). Since the negative glucocorticoid feedback effect was abolished, AP POMC-related peptides could have been depleted into peripheral blood; as a consequence, we found an increase in ACTH and β-END plasma levels at this time post-surgery. Something similar occurred in 2-day ADX rats; it would be reasonable to expect a similar effect in 2-day ENUC-rats; at this time-period after surgery, plasma B levels were significantly lower than those found in SHAM-operated rats.

There seems to be an inverse relationship between corticotrope hormone storage and release 2 days after ADX; while the output process was increased, peptide synthesis did not compensate for normal storage, as evidenced by the low AP hormone content (18). Later on, more than 2 days after ADX, the increased AP hormone content might be due to a large increase in POMC secretagogues secretion from the hypothalamus as a consequence of the lack of endogenous glucocorticoids (25, 26). Plasma ACTH levels increased in a time-dependent fashion from day 2 after ADX, reaching a 120-fold increase vs. SHAM-rats 21 days after ADX. These data on plasma ACTH levels tally with those reported by Dallman et al. 48 h after surgery (27) and with our previous results on days 7 and 14 after ADX (18). Similarly to ADX-rats, plasma ACTH concentrations increased markedly between 2 and 7 days after ENUC; however, unlike adrenalectomized-rats, they declined towards SHAM values by 21 days after ENUC. Assuming that adrenal regeneration was incomplete 7 days after ENUC (Ref. 3, 14 and Fig. 3C), it would be reasonable to expect high plasma ACTH concentrations at this particular time after ENUC (Fig. 1, upper panel). This observation tallies with elevated plasma ACTH levels previously reported 10 days after ENUC (15). Similarly, plasma β-END levels had the same pattern of ACTH during adrenal regeneration, as might be expected since both peptides derive from the same pro-hormone at the corticotrope level. The pattern of plasma ACTH and β-END levels correlates well with data of AP peptide content; ADX-rats showed a persistent increment in AP hormone content, reaching maximal values 7-14 days after surgery and then remaining at a high level (ACTH and β-END) or returning to SHAM values (β-END-LI) by 21 days post ADX. Although ENUC-rats showed a similar pattern of AP ACTH and β-ENDs to that observed in ADX-animals up to 7 (ACTH and β-END) or 14 (β-END-LI) days post surgery, the pattern was quantitatively different at 14 (ACTH and β-END) and 21 (ACTH, β-END and β-END-LI) days after surgery, the values in ENUC-rats being significantly higher than those in ADX-rats.

It has been established that the fall in peripheral glucocorticoid levels after ENUC is the main factor triggering the adrenal regenerative process; in fact, the administration of either adrenal extracts or corticosterone to ENUC-animals is able to fully interrupt adrenal regeneration (3, 4). The differences we found between ENUC and ADX conditions might be attributed to changes in plasma corticosteroid levels occurring during adrenal regeneration. Adrenal corticosterone output by regenerating glands restored SHAM-levels by 7 days after ENUC, thus clearly inhibiting corticotrope secretion by 21 days post-ENUC. Our results also indicate that, at least 14 days after ENUC, the negative glucocorticoid feedback mechanism seems to be not fully restored at higher levels of the central nervous system (e.g. hippocampus, hypothalamus) as indicated by the

increased storage of AP POMC-related peptides. We have recently observed (manuscript in preparation) that at 21, but not at 14, days after surgery the ratio vasopressin: CRH in the hypothalamus is significantly higher in ENUC than in ADX and SHAM animals, whereas the same ratio at the median eminence level is significantly higher in ADX than in ENUC and SHAM rats, thus, it could be expected that an enhanced input of POMC secretagogues on the AP at 14 days after ENUC has taken place (14). In addition, a lack of glucocorticoid effect on corticotrope synthesis during late adrenal regeneration (days 14 and 21 post-ENUC) tallies with previous *in vitro* studies carried out in normal corticotropes and in AtT20 cells; it was found that a decrease in POMC mRNA occurred only after 48 h of direct dexamethasone treatment (28). It remains to be determined whether a longer exposure of corticotropes to physiological levels of glucocorticoids is necessary to restore AP POMC-related peptides storage at the SHAM-level; this question is sustained by the observation of Birnberg et al. (29), who found increments in both plasma ACTH levels and AP POMC mRNA 2 days after ADX as well as in AP ACTH one week post surgery; they also found that dexamethasone treatment, in 8-day ADX rats, rapidly (between 2 and 8 h) reduced plasma ACTH levels and AP POMC mRNA, but that this treatment only decreased AP ACTH 8 days after administration. We cannot rule out a change in the ratio corticosterone: 18-hydroxydeoxycorticosterone in plasma, observed after ENUC (23), which could also affect AP function during adrenal regeneration.

It is noteworthy that, 21days after ADX, AP β-END-LI was similar to the respective SHAM-value; thus probably indicating the existence of a different source of POMC-related peptides belonging to the C terminal portion; however, this observation needs to be further investigated. Conversely, the same did not occur for AP ACTH and β-END 21 days post-ADX, since several fold higher values than in SHAM rats were found. These results are quite consistent with previous reports (18); they probably reflect that the lack of glucocorticoid induces an increase in AP ACTH and β-END synthesis and output due to an increased input of ME CRH and other secretagogues on the corticotropes (14).

In addition, we described the pattern of another small POMC-related peptide in the AP after ADX and ENUC: γ_3-MSH, which is normally produced by the intermediate lobe of the pituitary gland (30). POMC converting enzyme (17) and serine protease PC2 (31) process N-POMC$_{1-74}$ to N-POMC$_{1-48}$ and γ_3-MSH. We found AP γ_3-MSH changes in parallel with other AP POMC-related peptides after both ADX and ENUC; this observation agrees with previously reported data (13). It is important to point out that the production of N-POMC$_{1-48}$ by the AP after ENUC is essential for the following adrenal regeneration because of its mitogenic activity at the adrenal level (2). Is has been demonstrated that POMC cleaving enzymes process N-POMC$_{1-4}$ when the Thr$_{45}$ is not O-glycosilated (17, 32); thus, the increase in AP γ_3-MSH and N-POMC$_{1-48}$ production found after ENUC and ADX might indicate changes in the post-translational processing of N-POMC, such as O-glycosilation, and/or the activation of other unknown processing enzymes which could be modulating this process. It remains to be determinated the changes in circulating γ_3-MSH after both process once an immunoassay for direct plasma samples with no interference could be developed.

In summary, we found important changes in AP POMC-related peptides after ENUC and ADX, when compared to SHAM-rats. Moreover, the corticotrope activity was different when comparing between ENUC and ADX animals. In addition, AP ACTH and β-END were well correlated with their respective plasma levels after both types of surgery. As we described above, circulating adrenal glucocorticoids, during adrenal regeneration, were able to induce a faster negative feedback mechanism in AP POMC-related peptides secretion than in their synthesis. We conclude that these differences between ENUC and ADX might be due to changes in the nature of steroids secreted by regenerating adrenals (23). It remains to be determined whether particular steroids released during adrenal regeneration could be acting particularly in the hypothalamus, thus inducing changes in the synthesis and release of vasopressin/CRH, substances which in turn manage corticotrope activities during adrenal regeneration and after ADX.

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