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Hormonal Modulation of Apoptosis in the Rat Adrenal Gland In Vitro Is Dependent on Structural Integrity

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The intact rat adrenal gland in short-term (3-h) organ culture may be amenable for the identification of factors involved in regulating adrenal cell apoptosis under defined conditions. In this model, culturing in the absence of trophic support (basal; control) triggered apoptosis in the intact rat adrenal gland; oligonucleosome formation, a measure of apoptosis, was 56.4-fold greater than that of glands snap-frozen at the start of incubation. Angiotensin II (Ang II) (100 n*M*) enhanced apoptosis by 67% over control. By contrast, adrenocorticotropin (ACTH) (100 n*M*) attenuated basal apoptosis by 59% and antagonized the enhanced apoptosis induced by Ang II back to the control level. Quartering of the glands enhanced basal oligonucleosome formation 182.2% greater than that of intact glands. Interestingly, quartering of the glands abolished the influences of Ang II and ACTH on apoptotic DNA fragmentation, but did not alter ACTH-induced corticosterone secretion. These data suggest that some level of gross adrenal structural information or compartmentalization, sufficiently disrupted by quartering, is required for the hormonal modulation of adrenal cell survival.

Key Words: Adrenal gland; apoptosis; oligonucleosome formation; internucleosomal DNA fragmentation; adrenocorticotropin; angiotensin II; rat (Sprague-Dawley).

Introduction

The adrenal gland is essential for life. Postnatally, it is constantly undergoing remodeling to maintain the zonal composition of the cortex. Within certain limits, remodeling keeps in pace with mammalian growth to maintain optimal adrenal cortical mass *(1)*. This zonal composition in mammals tends to exhibit marked histological and functional heterogeneity, although recent evidence suggests the potential for both phenotypic and proliferative plasticity *(2–5)*.

Remodeling appears to involve proliferative potential, phenotypic plasticity, and apoptotic cell death. First, evidence suggests that the cells comprising each zone are members of the same cell lineage. However, the location of the stem cells is controversial: they may exist as a subcapsular blastema *(6)*, within the zona glomerulosa *(7,8)*, or at the glomerulosa/fasciculata boundary (zona intermedia) *(9)*. Cells entering the lineage are thought to undergo a centripetal "migration" (and concomitant differentiation) that ends at the most central position of the cortex, the zona reticularis abutting the medulla, where the cells ultimately degenerate and die *(6,7)*. However, this simplistic scheme may require modification. Other elegant work by Engeland and coworkers suggests that in addition to progenitor cells, more phenotypically differentiated cells within the zona fasciculata have proliferative potential in response to experimental perturbations, such as adrenal regeneration in response to enucleation *(3)* and compensatory adrenal growth in response to unilateral adrenalectomy *(4)*. Second, cells within the zonae glomerulosa and fasciculata exhibit phenotypic plasticity in response to tropic hormone (i.e., adrenocorticotropin [ACTH] *[5]*) and low sodium diet *(3,4)* and in response to experimental perturbations, such as adrenal regeneration in response to enucleation *(2,3)* and compensatory adrenal growth in response to unilateral adrenalectomy *(4)*. Third, although many cells die in the zona reticularis, evidence suggests that about half of the cells die en route during centripetal migration *(6)*. Thus, it appears that adrenocortical cells are constantly "culled" from the cortex during remodeling for optimal zonal proportion.

Although it seems that these components of adrenocortical remodeling are differentially recruited in response to growth-promoting and homeostatic perturbation stimuli,

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little is known about the role of the cell death component. Evidence suggests that the type of cell death that occurs in the adrenal cortex is apoptosis *(10)* and entails DNA fragmentation *(11,12)*. Since about half of the cells die en route during centripetal migration *(6)*, it appears that adrenocortical cells acquire the apoptotic machinery early in the lineage. However, in vivo, after ACTH withdrawal (i.e., hypophysectomy), apoptosis is restricted to a subpopulation of zona reticularis cells adjacent to the medulla *(12)*. How this apoptotic machinery is regulated by ACTH, angiotensin II (Ang II), other pituitary factors, the adrenal innervation, and intraglandular factors is unclear. In addition, the role of tissue organization and parenchymal cytoarchitecture, which serve as a framework through which these regulating components operate, is unknown.

In our previous work *(12)*, we established an in vitro model, viz., rat adrenal glands in short-term organ culture, to study the regulation of adrenal apoptosis under defined conditions. Culturing of intact rat adrenal glands in the absence of trophic support results in robust oligonucleosome (internucleosomal DNA cleavage) formation, a marker of apoptosis *(13–17)*. The purpose of the present study was to validate further this in vitro system as a means to identify the factors, signals, and mechanisms regulating adrenal cell survival. In addition, as a beginning point of these investigations, we addressed the unknown role of gross structural integrity of the adrenal gland. Herein we report the acute influence of two prominent adrenal steroidogenic hormones, ACTH and Ang II, on adrenal apoptosis. In addition, we demonstrate that adrenal structural integrity is obligatory for hormonal regulation of adrenal apoptosis, but not for hormonal stimulation of corticosterone production.

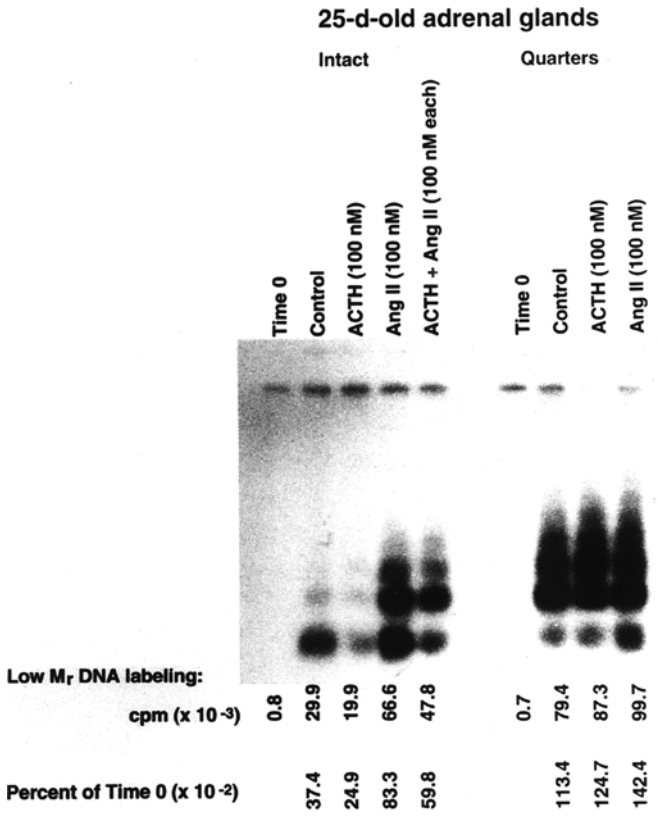

Fig. 1. Internucleosomal DNA fragmentation in cultured intact adrenal glands and adrenal gland quarters from 25-d-old male rats. This is a representative autoradiogram of oligonucleosome formation in the rat adrenal gland. Adrenal glands or adrenal gland quarters were either immediately snap-frozen at the start of incubation or were incubated without (control) or with the indicated concentrations of hormones for 3 h. Respective fractions of labeled low-M_r DNA fragments (<10 kb) were excised from gels and counted in a β-counter. Below the autoradiogram is the corresponding quantification of the labeled low-M_r DNA fragments.

Results

Internucleosomal DNA Fragmentation in Cultured Intact Adrenal Glands

The pattern of oligonucleosome formation, a characteristic of apoptotic cell death, but not necrosis *(13–17)*, was similar for adrenal glands from 25-d-old and 42-d-old rats (Figs. 1 and 2). Culturing in the absence of trophic support (control) triggered robust oligonucleosome formation. After 3 h of incubation, the increase in labeling of low-M_r (<10 kb) DNA fragments was 56.4 ± 19.7-fold greater than that at time 0 (mean ± SE, n = 4).

The inclusion of ACTH (100 n*M*) at the start of incubation attenuated culture-dependent oligonucleosome formation. Low-M_r DNA labeling in the presence of ACTH was 59.1 ± 17.3% less than that of the control (mean ± SE, n = 3).

Interestingly, the inclusion of Ang II (100 n*M*) at the start of incubation enhanced oligonucleosome formation. Low-M_r DNA labeling in the presence of Ang II was 113.1 ± 24.4% greater than that of the control (mean ± SE, n = 3).

ACTH consistently attenuated the apoptotic influence of Ang II in that low-M_r DNA labeling in the presence of equal concentrations of ACTH and Ang II (100 n*M* each) was 53.2 ± 13.6% less than that in the presence of Ang II alone (mean ± SE, n = 3). Conversely, Ang II attenuated the antiapoptotic effect of ACTH in that low-M_r DNA labeling in the presence of both hormones was not significantly different from that of the control (–11.3 ± 13.9% vs control [mean ± SE, n = 4]).

Internucleosomal DNA Fragmentation in Cultured Adrenal Gland Quarters

Here again, the pattern of oligonucleosome formation was similar for adrenal gland quarters from 25- and 42-d-old rats (Figs. 1 and 2). However, the differences in oligonucleosome formation and the influence of ACTH and Ang II on oligonucleosome formation between intact glands and gland quarters were markedly apparent. In the absence of trophic support, culture-triggered oligonucleosome formation of adrenal gland quarters was markedly

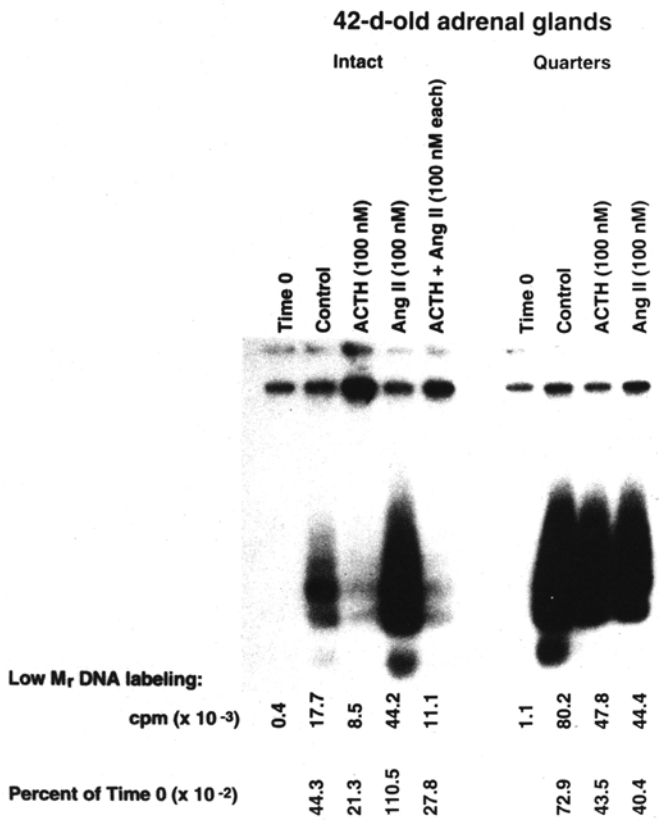

Fig. 2. Internucleosomal DNA fragmentation in cultured intact adrenal glands and adrenal gland quarters from 42-d-old male rats. This is a representative autoradiogram of oligonucleosome formation in the rat adrenal gland. Adrenal glands or adrenal gland quarters were either immediately snap-frozen at the start of incubation or were incubated without (control) or with the indicated concentrations of hormones for 3 h. Respective fractions of labeled low-M_r DNA fragments (<10 kb) were excised from gels and counted in a β-counter. Below the autoradiogram is the corresponding quantification of the labeled low-M_r DNA fragments.

greater than that of intact glands. After 3 h of incubation, the increase in labeling of low-M_r (<10 kb) DNA fragments of control quarters was 182.2 ± 58.8% greater than that of control intact glands (mean ± SE, $n = 4$).

Surprisingly, the aforementioned contrasting influences of ACTH and Ang II on internucleosomal DNA fragmentation in intact glands were abolished by quartering the glands. Low-M_r DNA labeling in the presence of ACTH or Ang II was not significantly different from that of the control (−11.2 ± 8.4% vs control [mean ± SE, $n = 4$]).

Corticosterone Secretion by Cultured Intact Adrenal Glands or Adrenal Gland Quarters

Corticosterone release was monitored in order to provide an additional comparative end point of hormonal influences on intact adrenal glands and adrenal gland quarters. Both intact glands and gland quarters released corticosterone over the 3-h incubation (Table 1). Irrespective of rat age, ACTH (100 n*M*) consistently stimulated corticosterone release from both intact glands and gland quarters. With intact adrenal glands, the amount of corticosterone released in response to ACTH was 94.5% greater than that of the control. With gland quarters, the effect of ACTH was greater than with intact glands: the amount of corticosterone released in response to ACTH was 147.2% greater than that of the control.

Compared to ACTH, the effect of Ang II (100 n*M*) on corticosterone release was modest and inconsistent. It modestly, but significantly increased corticosterone release from intact 42-d-old glands (29% greater than control), but not from intact 25-d-old glands. In addition, Ang II attenuated the effect of ACTH on intact glands from rats of both ages ($P < 0.05$). However, quartering of the glands abolished any effect of Ang II on corticosterone release.

Table 1
Corticosterone Secretion by Intact Rat Adrenal Glands or Adrenal Gland Quarters in Short-Term Organ Culture

Treatment	25-d-old intact gland, ng/gland[a]	42-d-old intact gland, ng/gland[a]
Time 0	3.4 ± 0.6	6.0 ± 1.2
Control	590.8 ± 91.8	634.0 ± 47.6
ACTH (100 n*M*)	1217.4 ± 54.4[c]	1159.8 ± 70.4[c]
Ang II (100 n*M*)	607.6 ± 68.4	816.4 ± 82.0[c]
ACTH + Ang II[b]	785.0 ± 136.8	974.6 ± 95.0[c]
Treatment	**25-d-old gland quarter, ng/gland quarter[a]**	**42-d-old gland quarter, ng/gland quarter[a]**
Time 0	12.4 ± 1.0	16.4 ± 3.6
Control	204.2 ± 50.0	393.4 ± 84.8
ACTH (100 n*M*)	520.0 ± 86.4[c]	942.8 ± 177.4[c]
Ang II (100 n*M*)	225.2 ± 60.8	356.0 ± 97.2

[a]An intact adrenal gland or a quarter of a gland was either immediately snap-frozen just prior to the start of incubation (time 0) or was incubated without (control) or with the indicated concentrations of hormones for 3 h. At the end of the incubation period, intact adrenal glands or adrenal gland quarters were snap-frozen for subsequent biochemical analysis of DNA integrity. The culture medium was frozen (−30°C) until radioimmunoassay for corticosterone (*see* Materials and Methods). Data are the means ± SE, $n = 8$.

[b]ACTH and Ang II were each added at a concentration of 100 n*M*.

[c]$P < 0.05$ vs corresponding control value.

Discussion

In the present study, we have demonstrated that the intact rat adrenal gland in short-term organ culture can serve as a model for identifying factors involved in adrenal cell survival while maintaining tissue architecture and cell-to-cell interaction. We have shown that the cultured intact rat adrenal gland undergoes prominent oligonucleosome

formation (internucleosomal DNA fragmentation) (Figs. 1 and 2), a characteristic of apoptotic cell death, but not necrosis *(13–17)*.

We postulate that total removal of the adrenal gland from the rat followed by culturing of the gland without trophic support provides a widespread nonspecific trigger of apoptotic cell death. By contrast, it appears that apoptosis in vivo after ACTH withdrawal (i.e., hypophysectomy *[12]* or prednisolone acetate suppression of pituitary ACTH *[10]*) is more finely regulated and restricted to a subpopulation of zona reticularis cells *(12)*. Compared to the low level of apoptosis in vivo following ACTH withdrawal *(12)*, the extensive internucleosomal cleavage in vitro observed here suggests that this fine regulation is lost or surmounted when the adrenal gland is removed from the animal and cultured without trophic support.

As might have been expected, ACTH exhibited strong antiapoptotic activity in this in vitro model. However, an intriguing finding was the strong apoptotic activity of Ang II. The reciprocal counteraction of these two hormones is very apparent in cultured intact adrenal glands (Figs. 1 and 2). The type 2 Ang II receptors, recently demonstrated to mediate apoptosis in some cell lines *(18)*, are most concentrated in the rat adrenal medulla *(19)*; however, these receptors exist in much lower, but significant concentrations throughout the adrenal cortex as well *(19,20)*. Similarly, ACTH receptors are found throughout the rat adrenal cortex, and interestingly, the concentration of receptors in zona glomerulosa cells is about eight times that in zona fasciculata/reticularis cells *(21)*. Thus, there is the potential for the interaction of ACTH and Ang II in the regulation of cell survival, especially in the adrenal cortex.

Presumably, hormonal influences on adrenal cell apoptosis occur via signals that are different from those associated with corticosteroid synthesis in that the antiapoptotic action of ACTH, but not ACTH-induced corticosterone secretion, was abolished by quartering of the gland (Figs. 1 and 2; Table 1). Quartering also appeared to abolish the effect Ang II on oligonucleosome formation; however, quartering may have maximized basal oligonucleosome formation precluding any further enhancement by Ang II.

The paradoxical increase in basal oligonucleosome formation and the abolishment of the antiapoptotic effect of ACTH by quartering without attenuating ACTH-induced corticosterone secretion suggest that some level of gross adrenal structure provides a unique signal(s) that puts a brake on untrammeled apoptosis in response to loss of trophic support and mediates the antiapoptotic actions of ACTH. Alternatively, a compartmentalized potent apoptotic factor, release by quartering, supervenes the antiapoptotic actions of ACTH. These hypotheses of the factors, signals, and mechanisms regulating adrenal cell survival are currently under investigation.

Materials and Methods

Adrenal Gland Culture

Adrenal glands were collected from 25- and 42-d-old male Sprague-Dawley rats. All procedures with rats were carried out in accordance with the "*Guide for the Care and Use of Laboratory Animals*," and were reviewed and approved by the Institutional Animal Care and Use Committee (ORA #0931-I-01). The glands were quickly trimmed free of adhering fat and connective tissue. To avoid the influence of animal variation on adrenal response in vitro, paired treatments were conducted with the adrenals from one rat as follows: time 0, ACTH; control, Ang II. Another rat was used for ACTH + Ang II. The remaining adrenal gland from this rat was quartered by bisecting the gland through the venous hilus and then bisecting the halves through their maximal points of thickness using fine dissecting scissors. This was done to yield quarters of nearly equivalent size and tissue composition for four treatments: time 0, control, ACTH, and Ang II. This experimental design was rigidly adhered to for subsequent replicate experiments. Each intact gland or gland quarter was placed in a well of a six-well multiwell tissue-culture plate (Falcon Primaria, #3846; Becton Dickinson and Co., Lincoln Park, NJ) (i.e., 1 gland or 1 quarter of a gland/well) containing 2 mL of serum-free Eagle's Minimum Essential Medium supplemented with sodium pyruvate (2 m*M*) and L-glutamine (2 m*M*), and containing penicillin (100 U/mL), streptomycin (100 μg/mL) (Sigma Chemical Co., St. Louis, MO), and 0.1% fatty acid-free BSA (Boehringer Mannheim Corp., Indianapolis, IN). Additions to the incubation medium were medium alone (control), human ACTH-(1-39) (ACTH) (100 n*M*), [Ile^5]Ang II (100 n*M*), and ACTH + Ang II (100 n*M* each) (all peptides from Peninsula Laboratories, Inc., Belmont, CA). Intact glands or gland quarters were incubated for 3 h under standard culture conditions (37°C; 95% air:5% CO_2) with gentle rocking. Following incubation, adrenal glands or quarters were snap-frozen (dry ice–ethanol bath) and then stored frozen (–80°C) until processing for autoradiographic analysis of DNA integrity *(17)*. Intact glands or gland quarters that were immediately snap-frozen at the start of incubation served as time 0 data points. Incubation medium was frozen (–30°C) until radioimmunoassay for corticosterone.

Biochemical Analysis of DNA Integrity

The qualitative and quantitative analyses of mononucleosomes and oligonucleosomes resulting from the internucleosomal DNA fragmentation that occurs during apoptosis were performed using microscale autoradiographic methods described in detail elsewhere *(17)*. In brief, total genomic DNA was extracted from frozen rat adrenal tissue using procedures that minimized mechanical fragmentation of DNA. Next, 3'-ends of apoptotic DNA fragments contained in sample DNA (1 μg) were labeled using

terminal deoxynucleotidyl transferase enzyme (calf / thymus; Boehringer Mannheim Corp., Indianapolis, IN) and [α^{32}P]-ddATP (DuPont NEN, Boston, MA) under conditions that ensured that only a single molecule of ddATP was incorporated per 3'-end at near-maximal efficiency. Labeled DNA was then fractionated by gel electrophoresis (250 ng/lane). The gel was dried and subjected to autoradiographic analysis. Incorporation of radiolabel into low-mol-weight (M_r) DNA (<10 kb) was quantitated by cutting the respective fraction of DNA from the dried gel, solubilizing it, and counting it for 1 min in a β-counter.

Radioimmunoassay for Corticosterone

Frozen incubation media were thawed at 4°C and then heated at 95°C for 5 min. Radioimmunoassay was performed directly on aliquots of incubation media using a commercially available kit (Diagnostic Products, Inc., Los Angeles, CA).

Data Analysis

Where appropriate, data were analyzed by Student's *t*-test (control vs treatment). Means were deemed significantly different when $P < 0.05$.

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