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Research Article

The effects of corticotropin-releasing hormone (CRH) and dexamethasone on proopiomelanocortin (POMC) mRNA levels in cultured pituitary adenoma cells were studied in 10 patients with Cushing's disease. As a control, POMC mRNA levels in cells from nonadenomatous tissues were examined in four patients. Human POMC mRNA in the cells was analyzed by Northern blot hybridization. Human POMC DNA probe hybridized with only a single size class of RNA (approximately 1,200 nucleotides) from the adenoma and nonadenoma cells of each patient. The size of POMC mRNA did not change through the culture or after incubation with CRH or dexamethasone. CRH increased POMC mRNA levels in these cells in a dose- and time-dependent manner. The minimum concentration of CRH required to elevate POMC mRNA levels in these cells exposed for 15 h was 0.1 nM. The minimum duration of 1 nM CRH treatment required to increase these levels was 3 h under our conditions. Inhibitory effects of 1 and 10 micrograms/dl dexamethasone on ACTH release and POMC mRNA levels in nonadenoma cells were greater than those in adenoma cells. These results suggest the following: (a) that the mRNA in cultured pituitary adenoma cells is qualitatively the same as that in vivo; (b) that responses of mRNA levels to CRH are time- and dose-dependent; and (c) that adenoma cells resist the inhibitory effect of […]

Effects of Corticotropin-releasing Hormone and Dexamethasone on Proopiomelanocortin Messenger RNA Level in Human Corticotroph Adenoma Cells In Vitro

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Abstract

The effects of corticotropin-releasing hormone (CRH) and dexamethasone on proopiomelanocortin (POMC) mRNA levels in cultured pituitary adenoma cells were studied in 10 patients with Cushing's disease. As a control, POMC mRNA levels in cells from nonadenomatous tissues were examined in four patients. Human POMC mRNA in the cells was analyzed by Northern blot hybridization. Human POMC DNA probe hybridized with only a single size class of RNA (~ 1,200 nucleotides) from the adenoma and nonadenoma cells of each patient. The size of POMC mRNA did not change through the culture or after incubation with CRH or dexamethasone. CRH increased POMC mRNA levels in these cells in a dose- and time-dependent manner. The minimum concentration of CRH required to elevate POMC mRNA levels in these cells exposed for 15 h was 0.1 nM. The minimum duration of 1 nM CRH treatment required to increase these levels was 3 h under our conditions. Inhibitory effects of 1 and 10 μg/dl dexamethasone on ACTH release and POMC mRNA levels in nonadenoma cells were greater than those in adenoma cells. These results suggest the following: (*a*) that the mRNA in cultured pituitary adenoma cells is qualitatively the same as that in vivo; (*b*) that responses of mRNA levels to CRH are time- and dose-dependent; and (*c*) that adenoma cells resist the inhibitory effect of dexamethasone on POMC mRNA levels and ACTH release.

Introduction

Hypothalamic corticotropin-releasing hormone (CRH)[1] is highly potent in stimulating the release and synthesis of proopiomelanocortin (POMC, the precursor to ACTH and endorphin) -derived peptides (1, 2). Furthermore, CRH increases POMC mRNA levels in rat anterior pituitary (AP) in vivo (3, 4) and in vitro (5, 6), and in mouse pituitary tumor cells (7) by increasing the rate of POMC transcription (8). On the other hand, glucocorticoids inhibit release of POMC-derived peptides (9), and decrease POMC mRNA levels in rat AP in vivo (8, 10, 11) and in mouse pituitary tumor cells (12, 13) by decreasing transcription of the POMC gene (8, 10, 11). There is little information available regarding the effects of CRH and glucocorticoids on POMC mRNA levels in human tissues. We have previously reported the effects of CRH and glucocorticoids on ACTH release from the pituitary glands of patients with Cushing's disease in vitro using a perifusion system (14, 15).

In the present study, we examined in detail the dose- and time-related effects of CRH and dexamethasone on ACTH release, and the steady state POMC mRNA levels in cultured pituitary adenoma cells of patients with Cushing's disease.

Methods

Pituitary cell culture. The pituitary macroadenomatous tissues were obtained from 10 patients and nonadenomatous tissues were obtained from four patients with Cushing's disease who had microadenomas at transsphenoidal surgery. The tissues were dissected and suspended in sterile Hepes buffer containing 1.4% collagenase (Cooper Biochemical, Malvern, PA), 0.04% dispase (Sanko Pure Chemical, Tokyo, Japan), and 2% BSA for 40 min at 37°C. They were then resuspended in Hepes buffer containing 0.002% DNase (Sigma Chemical Co., St. Louis, MO), and 2% BSA for 10 min. A 5-min digestion of the tissue was followed by two washes with Hepes-buffered DME supplemented with 10% FCS, 10 μg/ml streptomycin, and 100 U/ml penicillin G. Aliquots of 5×10^5 cells were placed in multiple-well dishes in 4 ml DME and were incubated at 37°C in humidified 95% air-5% CO_2 for 3 d. Three dishes were prepared for each point. Subsequently, cells were washed three times with 4 ml of DME and incubated with CRH or dexamethasone in 4 ml of DME supplemented with 0.1% BSA for 15 h. After the incubation, the media were removed and ACTH levels in the media were determined by RIA.

Isolation and Northern blot analysis of RNA. The cells were washed twice with 4 ml of ice-cold PBS and were scraped with a rubber policeman. Total cellular RNA was isolated by the guanidine/hot phenol method (16). Total RNA was quantified by ultraviolet absorption at 260 nm. 2-μg RNA samples were denatured with 1 M glyoxal and 50% DMSO and electrophoresed on a 1.4% agarose gel in 10 mM sodium phosphate buffer, pH 7.0. After electrophoresis, RNA was transferred to Hybond N (Amersham Corp., Arlington Heights, IL), dried, and then heated in a vacuum oven at 80°C for 2 h before hybridization. POMC mRNA affixed to the filter was hybridized to a 1.1-kb Sma I restriction fragment containing the entire third exon of the POMC gene (17) (a gift from Dr. J. Shine [California Biotechnology, Inc.] and Dr. J. L. Roberts [Mount Sinai Medical Center, New York, NY]). This POMC DNA fragment was radiolabeled with (α-^{32}P) deoxyadenosine ATP to a specific activity of $1.0–1.5 \times 10^8$ cpm/μg DNA by a nick-translation method. Prehybridization was performed at 65°C for 4 h with 50 ml of prehybridization buffer with the following composition: 6× SSC (1× SSC = 150 mM sodium chloride and 15 mM sodium citrate, pH 7.4), 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% BSA), 0.1% SDS, and 100 μg/ml of denatured salmon sperm DNA. In addition, the hybridization buffer contained 5×10^6 pm/5 ml of ^{32}P-labeled probe and 10 mM EDTA. At the end of incubation, the filter was washed at room temperature once in 2× SSC with 0.5% SDS and in 2× SSC with 0.1% SDS, and twice at 65°C in 0.1× SSC with 0.1% SDS. The filter was then exposed to a Kodak

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1. *Abbreviations used in this paper:* AP, anterior pituitary; CRH, corticotropin-releasing hormone; POMC, proopiomelanocortin.

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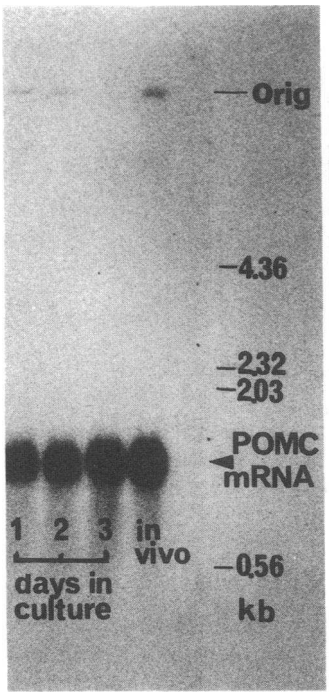

Figure 1. An example of Northern blot of POMC mRNA in 2 μg of total RNA extracted from cultured adenoma cells of a patient with Cushing's disease. DNA size markers: λ DNA was digested with Hind III, end-labeled, and denatured in the presence of glyoxal before electrophoresis.

XAR-5 x-ray film at −70°C for 1 (adenoma cells) or 14 d (nonadenoma cells), and the results of autoradiogram were quantified by scanning densitometry.

RIA. ACTH concentration in the medium was determined in duplicate by RIA as previously described (14, 15). Synthetic human ACTH and ACTH antiserum were supplied by the National Hormone and Pituitary Program, National Institute of Diabetes, Digestive, and Kidney Disease.

Statistics. Relative POMC mRNA levels are expressed as a percentage of the control. The data were statistically evaluated by one-way analysis of variance.

Results

The POMC DNA probe hybridized with only a single size class of mRNA (~ 1,200 nucleotides), from the adenoma and nonadenoma cells in all patients, and this result agrees with the reported size of human POMC mRNA (17, 18). The size of POMC mRNA did not change through the culture or after incubation with CRH or dexamethasone (Fig. 1). The ratio of POMC mRNA to total RNA, however, decreased to 40% of that in vivo (without dispersion) after a 1-d culture, then increased and reached a maximum (80% of in vivo data) after a 3-d culture (Fig. 1). There was not a significant difference in POMC mRNA levels in the cells between the 3- and 4-d cultures (data are not shown).

CRH treatment. In the time-course study, the increase in both POMC mRNA levels in adenoma cells and ACTH levels in the medium depended on the length of exposure of the cells to CRH (Fig. 2). Significant increases in ACTH levels in the medium were observed after 1 h of 1 nM CRH treatment (Fig. 2 *A*). ACTH secretion from adenoma cells after a 15-h incubation without CRH was remarkably higher than that from nonadenoma cells (Table I). POMC mRNA levels did not change during a 15-h incubation without CRH, but increased significantly after 3 h of CRH treatment (135±6% of control, mean±SE) with an ~ 2.5-fold elevation seen after 15 h of exposure (Fig. 2 *B* and *C*).

Dose-related increases in POMC mRNA levels in adenoma and nonadenoma cells and ACTH levels in the media were observed after 15 h of CRH treatment (Fig. 3). Significant increases in POMC mRNA levels were observed as a result of 0.1 nM (adenoma, 186±30%; nonadenoma, 130±8% of control) and 1 nM (adenoma, 280±75%; nonadenoma, 200±20% of control) CRH treatment, whereas ACTH levels in the medium increased significantly with 0.01 nM CRH (adenoma, 125±20%; nonadenoma, 151±35% of control) treatment. There was a variety of ACTH release and POMC mRNA levels responded to CRH in the cells. Therefore, when the effect of CRH on adenoma and nonadenoma cells are compared, there was not a significant difference between the two groups.

Dexamethasone treatment (Fig. 4). There was also a variety of ACTH release, and POMC mRNA levels responded to dexamethasone in adenoma cells. In nonadenoma cells, ACTH release and POMC mRNA levels were suppressed after 15 h of incubation with 1 and 10 μg/dl dexamethasone in a dose-dependent manner. ACTH release was 71±2% at 1 μg/dl

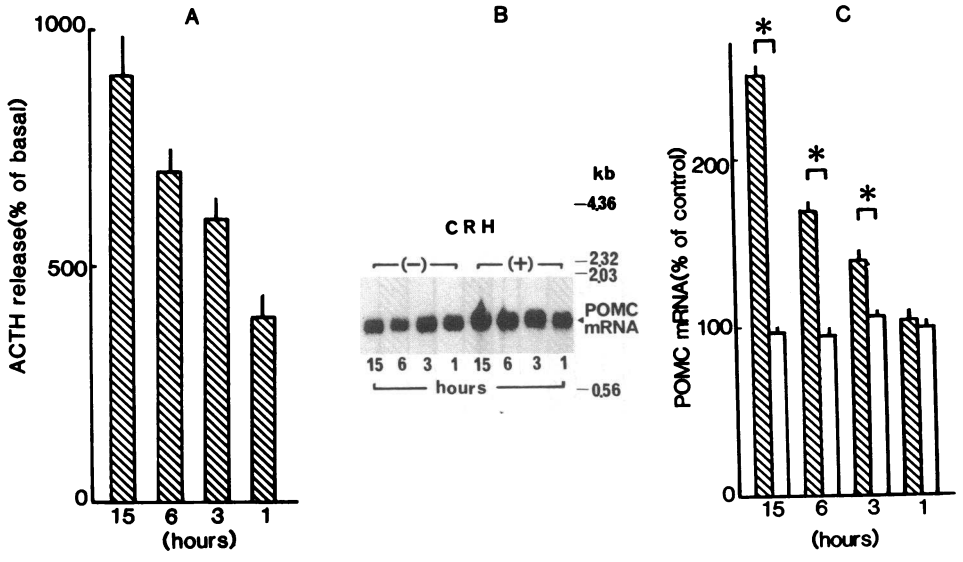

Figure 2. Time-dependent increases of ACTH levels in the media (*A*) and POMC mRNA levels (*B* and *C*). Adenoma cells from four patients were treated with (■) or without (□) 1 nM CRH. ACTH levels in the media (*A*) were expressed as percents of basal levels at each time. *B* is a representative Northern blot of POMC mRNA, and relative changes in POMC mRNA compared with the control (time 0) are depicted in *C*. *$P < 0.01$ vs. basal or control. Values are mean±SE.

Table I. ACTH Levels in the Medium

Adenoma cells*	Patient	ACTH
		ng/ml per 15 h
	1	281
	2	80
	3	92
	4	70
	5	123
	6	71
	7	42
	8	170
	9	231
	10	156
Mean±SE		131±24
Nonadenoma cells*	11	0.70
	12	0.65
	13	1.10
	14	0.91
Mean±SE		0.84±0.10

* 5×10^5 cells.

and 50±5% of control at 10 μg/dl; POMC mRNA levels were 93±1% at 1 μg/dl and 59±4% of control at 10 μg/dl. On the other hand, in adenoma cells, ACTH release and POMC mRNA levels were not inhibited by 1 μg/dl dexamethasone in all patients, but were inhibited by 10 μg/dl dexamethasone in 6 of 10 patients (ACTH release, 86±4%; POMC mRNA levels, 84±5% of control at 10 μg/dl). Therefore, ACTH release and POMC mRNA levels in nonadenoma cells were more suppressed by dexamethasone than those in adenoma cells ($P < 0.01$). When the time-course study on the effect of 10 μg/dl dexamethasone on POMC mRNA levels in adenoma cells from two patients was done, these levels did not decrease after the 6-h incubation, but did decrease after the 15-h incubation.

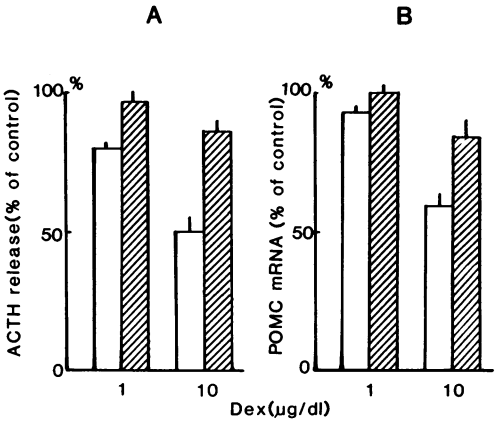

Figure 4. Effect of dexamethasone on ACTH levels in the media (*A*) and POMC mRNA levels (*B*) in adenoma (▨) and nonadenoma (□) cells. Values are mean±SE.

Discussion

In the present study, the ratio of POMC mRNA to total RNA decreased after a 1-d culture, then gradually increased and reached a maximum after a 3-d culture; these levels did not change during the 15-h incubation without serum. On the other hand, hybridization with the DNA probe revealed a single size class of POMC mRNA (~ 1,200 nucleotides) in adenoma cells in vivo (tissue fragments) and in vitro during both culture with serum and incubation without serum. The size of the POMC mRNA in adenoma and nonadenoma cells of these patients is the same as human pituitary POMC mRNA as previously reported (17, 18), and there was not a size heterogeneity of POMC mRNA, which was observed in ectopic ACTH-producing tumors (19–21) and extrapituitary tissues (22, 23). In addition, the present data suggest that the mRNA in adenoma cells during the culture is qualitatively the same as that in vivo. Therefore, a 3-d culture with serum is the best way to measure the levels of POMC mRNA in adenoma cells in vitro.

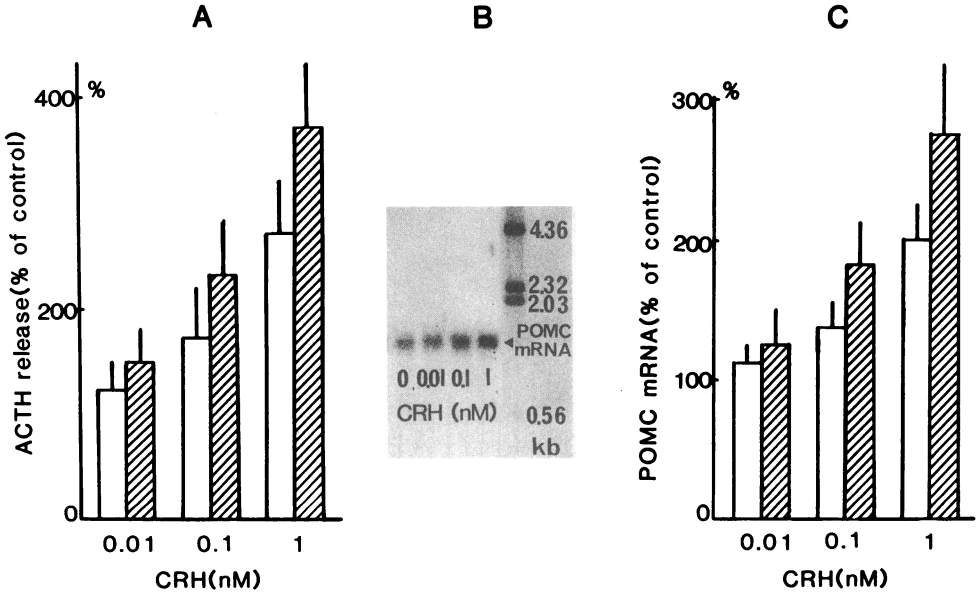

Figure 3. Dose-related increases in ACTH levels in the media (*A*) and POMC mRNA levels (*B* and *C*) in adenoma (▨, 10 patients) and nonadenoma (□, four patients) cells. The cells were treated for 15 h with different concentrations of CRH. *B* is an example of Northern blot of POMC mRNA in adenoma cells and *C* shows relative changes in POMC mRNA compared with the control. Values are mean±SE.

CRH increased both ACTH release and POMC mRNA levels dose- and time-dependently. CRH-induced increase in ACTH release occurred faster and was more sensitive than that in the POMC mRNA levels. These phenomena are also observed in rat AP; an increase in the transcription rate of POMC mRNA induced by CRH occurs within 30 min (8, 13, 23), whereas CRH-stimulated ACTH release occurs within 1 min (24, 25). Therefore, the discrepancy of the time lag between the increase in ACTH release and that in steady state POMC mRNA levels may be because the pool of cytoplasmic mRNA is quite large relative to the rate of new mRNA synthesis, therefore detectable alteration in cytoplasmic mRNA levels may be delayed (11, 26).

In the present study, there was a variety of responses of ACTH release and POMC mRNA to CRH in adenoma and nonadenoma cells. This result agrees with previous reports of a variety of CRH-induced ACTH release from the adenomas in vitro (14, 15) and plasma ACTH responses to CRH (27) in patients with Cushing's disease.

Basal levels of ACTH release and steady state POMC mRNA levels were decreased by 1 μg/dl dexamethasone in nonadenoma cells, but were not decreased in adenoma cells. In addition, in adenoma cells from 4 of 10 patients, neither ACTH release nor POMC mRNA levels was inhibited by 10 μg/dl dexamethasone. Therefore, pituitary adenomas seemed to be fairly sensitive to dexamethasone.

Glucocorticoids inhibit POMC mRNA transcription in the rat AP, with maximal inhibition occurring within 30 min (8, 10, 11). On the other hand, it takes > 6 h to decrease steady-state POMC mRNA levels in AP (10, 17). This may be due to a long half-life of POMC mRNA in mRNA in AP (18–24 h) (7, 10, 11, 13) and a large pool of POMC mRNA in the cytoplasma (13, 26).

In summary, (*a*) a single size class of POMC mRNA of ~ 1,200 nucleotides was detected in adenoma and nonadenoma cells of patients with Cushing's disease; (*b*) this POMC mRNA in the cultured cells is qualitatively the same as that in vivo; (*c*) CRH-stimulated increases of POMC mRNA levels were time and dose dependent; and (*d*) adenomas resist an inhibitory effect of dexamethasone on POMC mRNA levels and ACTH release.

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