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In Vivo and *In Vitro* Characterization of Antalarmin, a Nonpeptide Corticotropin-Releasing Hormone (CRH) Receptor Antagonist: Suppression of Pituitary ACTH Release and Peripheral Inflammation

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ABSTRACT

Corticotropin-releasing hormone (CRH) secreted from the hypothalamus is the major regulator of pituitary ACTH release and consequent glucocorticoid secretion. CRH secreted in the periphery also acts as a proinflammatory modulator. CRH receptors (CRH-R1, R2α, R2β) exhibit a specific tissue distribution. Antalarmin, a novel pyrrolopyrimidine compound, displaced ^{125}I-oCRH binding in rat pituitary, frontal cortex and cerebellum, but not heart, consistent with antagonism at the CRHR1 receptor. *In vivo* antalarmin (20 mg/kg body wt.) significantly inhibited CRH-stimulated ACTH release and carageenin-induced subcutaneous inflammation in rats. Antalarmin, or its analogs, hold therapeutic promise in disorders with putative CRH hypersecretion, such as melancholic depression and inflammatory disorders.

Corticotropin-releasing hormone (CRH) originating from the hypothalamus, is the primary physiological regulator of ACTH secretion from the anterior pituitary (1, 2). In addition, CRH is a potent stimulus of the arousal and autonomic nervous systems. Intracerebroventricular injections of CRH mimic the stress response in mice, rats, and nonhuman primates, both biologically and behaviorally. Central CRH hypersecretion has been inferred in human diseases, including melancholic depression and anorexia nervosa (2). CRH and its receptors have also been localized in peripheral tissues including immune (1,3-4), cardiovascular (5), and reproductive organs (6-9). Peripheral CRH, designated immune CRH, and secreted in inflammatory sites (10), plays a direct immunomodulatory role as an autocrine or paracrine mediator of inflammation. Immune CRH hypersecretion has been shown in autoimmune inflammatory diseases, including rheumatoid arthritis and Hashimoto thyroiditis (1).

Specific, seven transmembrane domain, G protein-coupled receptors bind to and mediate the actions of CRH both in the central nervous system (CNS) and periphery (11-18). Recently, two subtypes of the CRH receptors (CRHRI, CRHR2) were identified, cloned, and characterized in terms of their pharmacological specificity and regional localization in the rat (11-13), mouse (11, 14-16), chicken (17), and human (11,18). Both CRHR1 and CRHR2 are coupled to stimulation of adenylyl cyclase (11-18).

Peptide analogs of CRH have been identified which inhibit CRH binding to its receptor and antagonize its actions *in vitro* and *in vivo* (19,20).

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Very recently, nonpeptide CRH receptor antagonists were developed, which by their chemical nature could offer the advantages of oral administration, long duration of action, and activity within the CNS. We have recently prepared the antagonist antalarmin, originally developed by Chen (21), for use as a research tool to further investigate the physiologic central and peripheral roles of CRH.

MATERIALS AND METHODS

Synthesis of antalarmin: *N*-Butyl-*N*-ethyl-[2,5,6,-trimethyl-7-(2,4,6-trimethylphenyl)-*7H*-pyrrolo[2,3-d]pyrimidin-4-yl]amine was synthesized in 5 steps from 2,4,6-trimethylaniline, using modifications of the procedure reported by Chen *et al.*, 1994 (21). The pyrrolopyrimidine was purified by silica gel flash chromatography (eluent = hexane-ethyl acetate 5:1) affording the desired compound as an oil, which crystallized on standing (mp 70-72 °C). ^{1}H NMR (300MHz, $CDCl_3$, Varian XL-300) gave identical peaks to the published values. CIMS (Finnigan 1015 Mass spectrometer) gave the required M+1 peak), and C, H, N combustion analysis (Atlantic Microlabs, Atlanta, GA) was within ± 0.4% of calculated values. Product homogeneity was confirmed by thin-layer chromatographic analysis (single spot with 3 different solvent systems, Analtech Uniplate silica gel GHLF) and by gas chromatography (Hewlett-Packard 5890A Capillary GC, J & W DB5 column).

Experimental Animals: Adult (2-3 mo. old) male Sprague Dawley rats (Taconic Farms, Germantown, NY) were maintained on a 14-h light (lights on at 0600 h), 10 -h dark cycle, with food and water available *ad libitum*. For the receptor binding experiments, animals were decapitated and the tissues were rapidly dissected, frozen on dry ice, and stored at -70°C.

Receptor Binding Assay: Rat frontal cortex, pituitary, cerebellum, and heart tissue homogenates were incubated with ^{125}I-ovine CRH and increasing concentrations of antalarmin. Nonspecific binding was determined in every assay and defined as the amount of ^{125}I-ovine CRH bound in the presence of 1 μM rat/human CRH. Rat tissues were weighed and subsequently homogenized in 20 vol. of ice-cold homogenization buffer (PBS, 10 mM $MgCl_2$, 2 mM EGTA, pH 7.0). The binding assay proceeded essentially as previously described (3,7) with the following modification. The assay was terminated at the end of 120 min by the addition of 1 ml of 7.5% polyethylene glycol, and centrifugation at 14000 x g at 4°C for 15 min. The supernatant was aspirated and the pellet was washed with 1 ml of 0.01% Triton-X-100 in PBS. After an additional similar centrifugation, the supernatant was aspirated and the radioactivity of the pellet was measured in a gamma counter. Binding data were analyzed by LIGAND (22) to determine the Ki values.

Suppression of CRH-induced ACTH Release: In initial *in vivo* experiments examining CRH-induced ACTH release, rats were divided into 4 groups: vehicle + saline, vehicle + rat/human CRH (r/h CRH), antalarmin + r/h CRH, and CRH antiserum + r/hCRH. The rats were first injected intraperitoneally (ip) with 0.5 ml of vehicle solution, Liposyn II 20%. One-and-a-half hr after receiving vehicle, antalarmin, or CRH antiserum, rats were injected ip with 0.5 ml of saline or 4.74 ng of r/h CRH dissolved in saline. Thirty min later, the animals were decapitated and trunk blood was collected into tubes containing 15 mg EDTA. ACTH values were measured by RIA (Hazelton Laboratories, Vienna, VA).

Carrageenin-induced Subcutaneous Inflammation: This procedure was performed as previously described (10). In preliminary experiments examining the effects of antalarmin on carrageenin-induced air pouch inflammation, rats were divided into 3 groups and injected ip with vehicle, 20 mg/kg of antalarmin dissolved in Liposyn, or 1.0 ml CRH polyclonal antiserum. A full dose-response relation between antalarmin and suppression of inflammation was then obtained with 0, 2, 7, 15, 20 mg/kg of antalarmin dissolved in 0.5 - 0.6 ml of Liposyn.

RESULTS AND DISCUSSION

Antalarmin potently displaced ^{125}I-oCRH binding, exhibiting respectively Ki values of 1. 9 ± 0.9, 1.3 ± .4, and 1.4 ± .6 nM (mean ± SEM) in pituitary, cerebellum, and frontal cortex homogenates. Approximately 80% of the specific binding of ^{125}I-oCRH observed with 1μM r/h CRH, was displaced in pituitary, cerebellum, and frontal cortex homogenates (Fig. 1). Antalarmin did not antagonize specific binding of ^{125}I-oCRH to heart

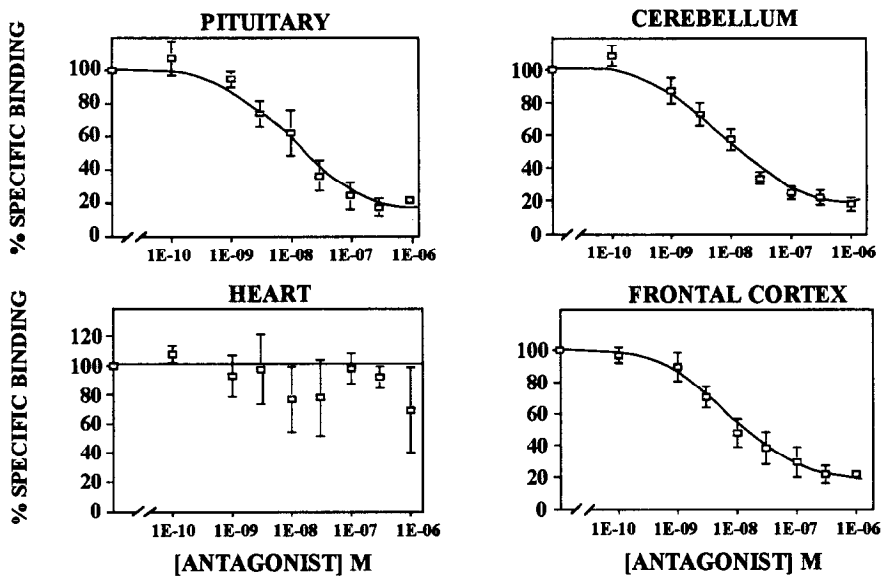

Figure 1. Inhibition of ^{125}I-oCRH binding in tissues differentially expressing CRH1 and CRH2 receptors. Tissue homogenates were incubated in the presence of approximately 0.6 nM ^{125}I-oCRH and increasing concentrations of antalarmin. Nonspecific binding was subtracted from the total binding. Data represent the mean + of duplicate determinations from 5-6 experiments.

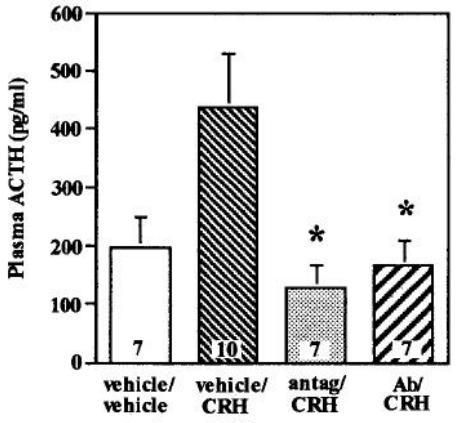

Figure 2. Inhibition of CRH-induced release of ACTH. The results shown were obtained from trunk blood collected 30 min after a CRH/saline injection in rats pretreated with ip vehicle, antalarmin., or anti-CRH. *$p \leq 0.05$ (ANOVA, Duncan multiple range). The number of animals in each group is included in the bars

homogenates. CRHR1 receptors are most abundant in neocortical, cerebellar, and sensory relay structures, and CRHR2 receptors are predominately localized in specific subcortical areas and peripheral tissues including the heart and arterioles (11). Antalarmin, by effectively displacing ^{125}I-oCRH binding in tissues predominately expressing CRHR1 but not in tissues expressing CRHR2, appears to be a specific CRHR1 receptor antagonist.

Antalarmin antagonized both central and peripheral actions of CRH *in vivo*. Indeed, the compound significantly suppressed CRH-induced ACTH secretion to approximately the same extent as neutralizing polyclonal anti-CRH (Fig. 2). Rats injected with 1 nmole (4.74 ng) of r/h CRH 1.5 h after the Liposyn vehicle or 20 mg/kg of antalarmin exhibited ACTH values of 436 ± 93 pg/ml and 126 ± 39 pg/ml, respectively (mean ± SEM). "Control" values of ACTH in rats injected with saline 1.5 hr after the Liposyn vehicle exhibited intermediate ACTH values of 167 ± 43 pg/ml. These data demonstrate that systemic administration of antalarmin blocks pituitary CRH receptors, and thus, exogenous or endogenous CRH-induced ACTH release. In the carrageenin-induced inflammation model, both antalarmin and the anti-CRH respectively suppressed the leukocyte concentration of subcutaneous exudate (Fig. 3A) by 36% and 27%. Statistical analysis using ANOVA followed by Dunnett's test, demonstrated a significant dose-dependent effect, which was significantly different from vehicle at the 2 mg/kg and 20 mg/kg doses, raising the possibility that the antagonist has a biphasic effect (see Fig. 3B).

We previously localized immunoreactive CRH (irCRH) in inflammatory sites in various experimental models of inflammation, including carrageenin-induced aseptic inflammation in Sprague-Dawley rats, and acute and chronic streptococcal cell wall- and adjuvant-induced arthritides in Lewis rats and RP-16-induced uveitis in Lewis rats and in B10.A mice (1, 10, 22, 24). Immune CRH appears to exert pro-inflammatory actions. Systemic administration of rabbit anti-CRH caused suppression of both the inflammatory exudate volume and cell concentration in carrageenin-

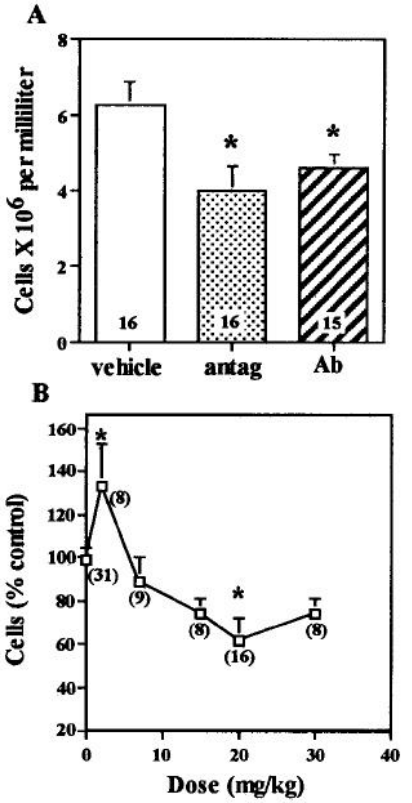

Figure 3. Suppression of carrageenin-induced subcutaneous inflammation. Panel A shows the leukocyte concentration of exudate collected from rats injected with either the vehicle,antalarmin, or 1 ml of anti-CRH ip prior to the subcuaneous injection of carrageenin. Panel B shows the leukocyte concentration, expressed as a percent of control, of the exudates from rats receiving the indicated dose of antalarmin. * $p \leq 0.05$, ANOVA, Duncan multiple range). The number of animals in each group is shown in the bars or parentheses.

induced inflammatory sites in rats (10) and ameliorated the severity of experimentally-induced uveitis in mice, when administered in the early stages of the disease(24). These results raise the possibility that pyrrolopyrimidine compounds, such as antalarmin, which antagonize CRH at the level of its own receptor, have therapeutic potential in some forms of inflammation.

The nonpeptide CRH antagonist tested here appears to be effective in blocking some central and peripheral actions of CRH in a CRHR1-specific fashion. Thus, antalarmin and its future analogs promise to enhance our understanding of the roles of CRH in many normal and pathologic states and to possibly provide a therapeutic tool for both CNS and inflammatory disorders associated with central and peripheral CRH hypersecretion, respectively.

References

1. Chrousos GP 1995 The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med 332:1351-1362
2. Chrousos GP, Gold PW 1992 The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 267:1244-1252
3. Webster EL, Tracey DE, Jutila MA, Wolfe SA,Jr., De Souza EB 1990 Corticotropin-releasing factor receptors in mouse spleen: identification of receptor-bearing cells as resident macrophages. Endocrinology 127:440-452.
4. De Souza EB, Grigoriadis DE, Webster EL 1991 Role of brain, pituitary and spleen corticotropin-releasing factor receptors in the stress response. Methods Achiev Exp Pathol 14:23-44.
5. Richter RM, Mulvany MJ 1995 Comparison of hCRF and oCRF effects on cardiovascular responses after central, peripheral, and in vitro application. Peptides 16:843-849.
6. Clifton VL, Owens PC, Robinson PJ, Smith R 1995 Identification and characterization of a corticotrophin-releasing hormone receptor in human placenta. Eur J Endocrinol 133:591-597.
7. Mastorakos G, Webster EL, Friedman TC, Chrousos GP 1993 Immunoreactive corticotropin-releasing hormone and its binding sites in the rat ovary. J Clin Invest 92:961-968.
8. Dufau ML, Tinajero JC, Fabbri A 1993 Corticotropin-releasing factor: an antireproductive hormone of the testis. FASEB J 7:299-307.
9. Makrigiannakis A, Margioris AN, Le Goascogne C, Zoumakis E, Nikas G, Stournaras C, Psychoyos A, Gravanis A 1995 Corticotropin-releasing hormone (CRH) is expressed at the implantation sites of early pregnant rat uterus. Life Sci 57:1869-1875.
10. Karalis K, Sano H, Redwine J, Listwak S, Wilder RL, Chrousos GP 1991 Autocrine or paracrine inflammatory actions of corticotropin-releasing hormone in vivo. Science 254:421-423.
11. Grigoriadis DE Lovenberg TW Chalmers D; Liaw C De Souze EB 1996 Characterization of corticotropin-releasing factor receptor subtypes. Ann N Y Acad Sci 780:60-80
12. Perrin MH, Donaldson CJ, Chen R, Lewis KA, Vale WW 1993 Cloning and functional expression of a rat brain corticotropin releasing factor (CRF) receptor. Endocrinology 133:3058-3061.
13. Lovenberg TW, Liaw CW, Grigoriadis DE, Clevenger W, Chalmers DT, De Souza EB, Oltersdorf T 1995 Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain Proc Natl Acad Sci U S A 92:836-840
14. Stenzel P, Kesterson R, Yeung W, Cone RD, Rittenberg MB, Stenzel-Poore MP 1995 Identification of a novel murine receptor for corticotropin-releasing hormone expressed in the heart. Mol Endocrinol 9:637-645.
15. Kishimoto T, Pearse RV, Lin CR, Rosenfeld MG 1995 A sauvagine/corticotropin-releasing factor receptor expressed in heart and skeletal muscle. Proc Natl Acad Sci U S A 92:1108-1112.
16. Perrin M, Donaldson C, Chen R, Blount A, Berggren T, Bilezikjian L, Sawchenko P, Vale W 1995 Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. Proc Natl Acad Sci U S A 92:2969-2973.
17. Yu J, Xie LY, Abou-Samra AB 1996 Molecular cloning of a type A chicken corticotropin-releasing factor receptor with high affinity for urotensin I. Endocrinology 137:192-197.
18. Chen R, Lewis KA, Perrin MH, Vale WW 1993 Expression cloning of a human corticotropin-releasing-factor receptor. Proc Natl Acad Sci U S A 90:8967-8971.
19. Curtis AL, Grigoriadis DE, Page ME, Rivier J, Valentino RJ 1994 Pharmacological comparison of two corticotropin-releasing factor antagonists: in vivo and in vitro studies. J Pharmacol Exp Ther 268:359-365
20. Gulyas J, Rivier C, Perrin M, Koerber SC, Sutton S, Corrigan A, Lahrichi SL, Craig AG, Vale W, Rivier J 1995 Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. Proc Natl Acad Sci U S A 92:10575-10579.
21. Chen YPL. International Patent number: WO 94/13676, June 23, 1994.
22. Munson PJ and Rodbard D 1980 Ligand: a versatile computerized approach for characterization of
Anal Biochem 107:220-39
23. Crofford LJ, Sano H, Karalis K, Webster EL, Goldmuntz EA, Chrousos GP, Wilder RL 1992 Local secretion of corticotropin-releasing hormone in the joints of Lewis rats with inflammatory arthritis. J Clin Invest 90:2555-2564.
24. Mastorakos G, Bouzas EA, Silver PB, Sartani G, Friedman TC, Chan CC; Caspi RR, Chrousos GP 1995 Immune corticotropin-releasing hormone is present in the eyes of and promotes experimental autoimmune uveoretinitis in rodents. Endocrinology 136:4650-4658.