Vol. 134, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1986 Pages 255-260

DEMONSTRATION THAT CORTICOTROPIN-RELEASING FACTOR BINDING TO RAT PERIPHERAL TISSUES IS MODULATED BY GLUCOCORTICOID TREATMENT IN VIVO AND IN VITRO

Jitendra R. Dave and Robert L. Eskay

Neurochemistry Section, Laboratory of Clinical Studies, National Institute on Alcohol Abuse and Alcoholism, DICBR, Bethesda, Maryland 20892

Received November 19, 1985

In a recent study we reported the presence of specific binding sites for corticotropin-releasing factor (CRF) in peripheral tissues of the rat (Endocrinology, 116, 2151, 1985). The objective of this study was to determine if CRF binding to peripheral tissues was modified following adrenalectomy and glucocorticoid replacement therapy. Adult male rats were adrenalectomized and CRF binding to liver, spleen and testicular membranes was determined at 5, 7 or 14 days following adrenalectomy. An additional group of adrenalectomized rats received subcutaneous injections of dexamethasone (75 µg/day) for 14 days. Adrenalectomy of rats for 14 days increased CRF binding to liver, kidney, testis, spleen and ventral prostate by approximately 65%-125% above sham-control values. CRF binding to membrane preparations obtained from the pancreas of sham-operated rats was undetectable; however, adrenalectomy produced detectable CRF binding in this tissue. Adrenalectomy produced a time-related increase in CRF binding to ventral prostate, spleen and liver tissue. Administration of dexamethasone to adrenalectomized animals prevented increased CRF binding to peripheral tissues observed following adrenalectomy alone. *In vitro* dexamethasone treatment of prostatic or hepatic homogenates from adrenalecomized rats resulted in a dose-related decrease in CRF binding activity. However, similar *in vitro* treatment of prostatic or hepatic homogenate with progesterone exhibited no significant effects on CRF binding. Our results suggest that glucocorticoids may be a regulator of peripheral CRF receptors.

Corticotropin-releasing factor (CRF), a hypothalamic regulatory peptide containing 41 amino acids, is known to be a potent stimulus for the release and synthesis of pro-opiomelanocortin (POMC)-derived peptides from the pituitary gland (1-5), such as ACTH, ß-endorphin (BE) and alpha-MSH. Furthermore, CRF also appears to exhibit extrahypophysiotropic effects, as evident by CRF-induced behavioral and electrophysiological changes after the intracerebroventricular administration of CRF (6-9). Recent studies have also demonstrated a wide extra-hypothalamic distribution of CRF-like immunoreactivity in the central nervous system (10- 13). The existence of CRF-like activity in a variety of peripheral tissues and tumors, including ACTH-producing colonic carcinoma, ectopic ACTH-producing tumors, lung carcinoma, adrenal gland, gut, and pancreas, has been previously demonstrated (14-18). High affinity binding sites for CRF in the anterior and intermediate lobes of the pituitary gland have been identified and characterized (19-21). Binding of CRF to pituitary-gland receptors appears to initiate the physiological actions of CRF, which includes stimulation of the adenylate cyclase-cAMP system. In recent studies, we demonstrated the presence of specific CRF-binding sites in a variety of peripheral tissues and erythrocytes and reported that activation of specific CRF-binding sites in adrenal tissue stimulates the adenylate cyclase-cAMP system (22,23). These observations suggest that CRF may have an important regulatory role in various peripheral

0006-291X/86 $1.50

tissues. CRF binding to rat anterior-pituitary membranes has been reported to decrease following adrenalectomy (19), which suggested that pituitary CRF receptors may be dependent on the presence of physiological amounts of adrenal steroids. Since the effect of glucocorticoids on CRF binding sites in peripheral tissues was not known, the objective of the present study was to determine if adrenalectomy and steroid replacement therapy produced changes in CRF binding to peripheral tissues.

METHODS

Rat CRF (rCRF) and rat tyrosinated CRF (r[Tyr^0]CRF) were purchased from Peninsula Laboratories (Belmont, CA) or Bachem (Torrance, CA). Adrenalectomized or sham-operated adult (200-250 g) male Sprague-Dawley rats were maintained on a 12-h light-dark cycle, with food and 0.9% NaCl solution available *ad lib.* A group of adrenalectomized rats received subcutaneous injections of 75 µg dexamethasone daily for 14 days. Adrenalectomized and sham-operated rats were decapitated 5, 7 or 14 days after the surgery and their liver, kidneys, testes, pancreas, ventral prostate, and spleen were immediately frozen in liquid nitrogen and subsequently pulverized. Each tissue was then homogenized (Polytron, Brinkmann Instruments, Westbury, NY) at a setting of 6 for 1 min at 4 C in approximately 10 vol 0.3 M sucrose buffered to pH 7.6 with 25 mM Tris-HCl. The supernatant fluid from a 20-min preliminary 15,000 x g centrifugation was recentrifuged at 100,000 x g for 1 h to obtain a membrane fraction (100 K g pellet). This membrane fraction was resuspended in 10 mM MgCl2-25 mM Tris-HCl at pH 7.6 to provide approximately 3 mg/ml protein (24). Rat tyrosinated CRF was iodinated with [^{125}I] (Amersham)by a modification of the lactoperoxidase method of Thorell and Johansson (25,26). One hundred microliters of membrane suspensions were incubated for 25 min at 4 C with 60,000-70,000 cpm [^{125}I]-labeled r[Tyr^0]CRF with or without unlabeled CRF in a final volume of 0.5 ml buffer (10 mM MgCl2-0.1% BSA-25 mM Tris-HCl, pH 7.6). Incubation was terminated by adding 1 ml chilled buffer to each reaction tube, followed by centrifugation at 3,200 RPM for 45 min. Pellets were washed with an additional 1 ml buffer and recentrifuged, and each pellet was counted in a Micromedic 10/600 gamma-counter (Horsham, PA). Each sample was assayed in triplicate. The number of specifically bound counts to each tissue was calculated as the difference between bound counts in the tubes containing 5 x 10^{-6}M or no unlabeled CRF. Routinely, 20-25% of total [^{125}I]-CRF added to each incubation tube was bound and 60-70% of these counts were specifically bound (i.e. displaceable by 5 x 10^{-6}M unlabeled CRF). Scatchard analysis was performed by incubating the iodinated CRF with varying amounts of unlabeled CRF (0 to 5 x 10^{-6}M)(27). Using 'LIGAND', a computer program system for fitting multiple sites (28), the data were analyzed and checked for the number of binding sites by Scatchard plot fit.

RESULTS

Specific binding of [^{125}I]-labeled r[Tyr^0]CRF to various peripheral tissues was rapid and time dependent at 4 C (data presented in Ref.22). Maximal binding was observed after 25 min of incubation, which declined steadily thereafter. The relative distribution of [^{125}I]-labeled rCRF binding to various peripheral tissue obtained from sham-operated controls is presented in Figure 1. Ventral prostate exhibited the highest density of CRF binding, followed by spleen, liver, kidney and testis. Pancreas obtained from sham-operated animals did not exhibit detectable CRF binding sites, whereas, pancreas obtained from adrenalectomized rats exhibited detectable levels of CRF binding sites (Figure 1). Fourteen days after adranectomy, CRF binding to peripheral tissue was consistently increased and the values, as percent of sham-operated controls, in ventral prostate, spleen, liver, kidney and testis were, 170%, 190%, 165%, 250% and 215%, respectively.

As shown in Figure 2, increased CRF binding following adrenalectomy was found to be time-dependent, at least in ventral prostate, spleen and liver. CRF binding was highest 14 days after adrenalectomy. Increased [^{125}I]-CRF

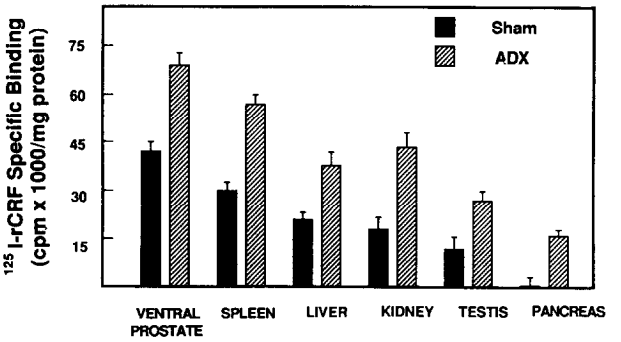

Figure 1: Demonstration that adrenalectomy of adult male rats increased ^{125}I-CRF binding to peripheral tissues. Animals were adrenalectomized (cross-hatched bars) or sham-operated (solid bars) for 14 days prior to sacrifice. ^{125}I-rCRF binding was carried out at 4 C for 25 min, as described in the text. Each bar represents the mean ± S.D. of 5-6 animals, assayed individually in triplicate.

binding to ventral prostate, spleen and liver in 14-day adrenalectomized rats was due to an increase in the number of high-affinity CRF binding sites. The apparent value of 'Q' (number of binding sites, pmol of rCRF/mg protein) increased from 15, 12 and 10 in sham-operated controls to 30, 26 and 22 in adrenalectomized rats for ventral prostate, spleen and liver tissue, respectively. The apparent binding affinity of CRF was not significantly affected after adrenalectomy (data not shown). The enhanced rCRF binding to ventral prostate, spleen and liver observed in adrenalectomized animals was reduced to that observed in control animals following dexamethasone treatment (subcutaneous injection of 75 µg/day for 14 days) of adrenalectomized animals (Figure 2).

To determine the effect of *in vitro* glucocorticoid treatment on [^{125}I]-rCRF binding, homogenates of prostate gland and liver obtained from adrenalectomized rats were treated with different concentrations of dexamethasone or

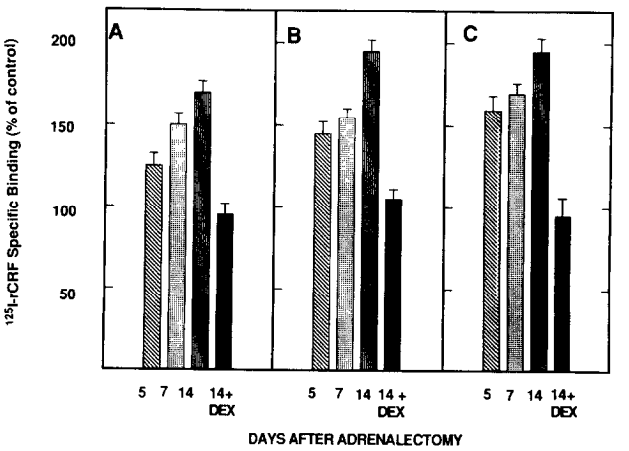

Figure 2: Demonstration that adrenalectomy of male rats produced a time-related increase in ^{125}I-rCRF binding to ventral prostate (A), spleen (B) and liver (C). A group of adrenalectomized animals received subcutaneous injections of 75 µg dexamethasone daily for 14 days (14 +DEX; solid bars). Each bar represents the mean ± S.D. of 5 animals, assayed individually in triplicate.

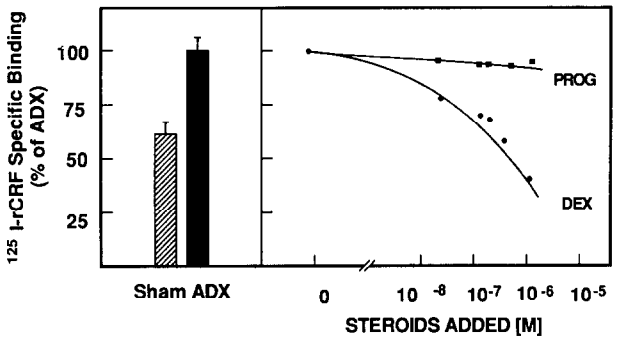

Figure 3: *In vitro* dexamethasone treatment of prostate gland homogenate obtained from adrenalectomized rats produced a dose-related decrease in CRF binding to the particulate fraction. A pool of 4 ventral prostate glands obtained from 14-day adrenalectomized rats was homoginized in 25 mM Tris-HCl, pH 7.6 containing 0.3 M sucrose, incubated with various concentrations of dexamethasone or progesterone for 10 min at 22 C, and centrifuged to obtain a particulate fraction, as described in the text. This membrane fraction was used for ^{125}I-rCRF binding as described in the text. Values are the mean of triplicate determinations from 1 of 2 experiments.

progesterone for 10 min at 22 C. After this *in vitro* treatment, membrane preparations were isolated and assayed for CRF binding activity. As demonstrated in Figure 3, *in vitro* treatment of prostatic homogenates with dexamethasone produced a dose-related inhibition of [^{125}I]-rCRF binding. Dexamethasone, at micromolar concentrations produced more than 60% inhibition of CRF binding to prostatic membranes, whereas, *in vitro* treatment of prostatic homogenates with progesterone exhibited no significant effects on CRF binding. Similarly, in vitro treatment of liver homogenates with dexamethasone produced a dose-related inhibition in CRF binding and micromolar concentration of dexamethasone inhibited CRF binding by more than 50% (Figure 4). *In vitro* treatment of liver homogenates with progesterone produced no significant effects on CRF binding. Direct addition

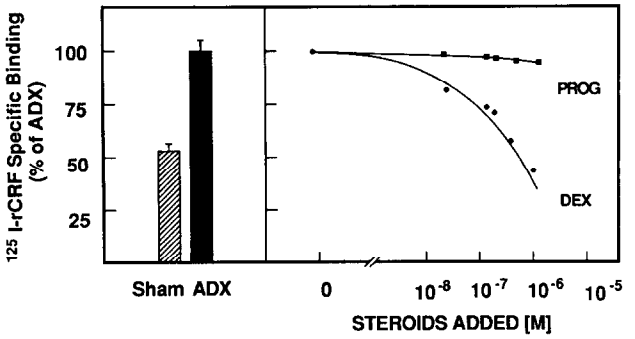

Figure 4: *In vitro* dexamethasone treatment of whole homogenate of liver obtained from adrenalectomized rats produced a dose-related decrease in CRF binding to the particulate fraction. A pool of 4 livers obtained from 14-day adrenalectomized rats was homogenized in 25 mM Tris-HCl, pH 7.6 containing 0.3 M sucrose, incubated with various concentrations of dexamethasone or progesterone for 10 min at 22 C, and centrifuged to obtain a particulate fraction as described in the text. This membrane fraction was used for ^{125}I-rCRF binding, as described in the text. Values are the mean of triplicate determinations from 1 of 2 experiments.

of micromolar concentration of dexamethasone to prostatic or hepatic membrane preparations produced less than a 10% inhibition of CRF binding (data not shown).

DISCUSSION

In an earlier study we reported that rCRF binding sites are present on membranes obtained from rat adrenal, ventral prostate, spleen, liver, kidney and testis. Occupancy of the peripheral CRF recognition sites, at least in adrenomedullary tissue, stimulates the adenylate cyclase- cAMP system, which suggests that the peripheral action of CRF may be mediated via the cAMP system (22). In the present study we report that adrenalectomy of adult male rats increases CRF binding to ventral prostate, spleen, liver, kidney and testis. Furthermore, administration of dexamethasone to adrenalectomized animals prevented this increase in CRF binding, at least in ventral prostate, spleen and liver. *In vitro* treatment of liver and prostate homogenates with dexamethasone produced a dose-related inhibition of CRF binding. However, *in vitro* treatment of liver and prostate homogenates with progesterone and *in vitro* treatment of liver and prostatic membranes with dexamethasone did not change CRF binding. These results suggest that glucocorticoids may be important modulators of CRF binding to peripheral tissue. Our findings are consistent with a recent report in which adrenalectomy increased prolactin receptors in liver (29). Furthermore, addition of dexamethasone and corticosterone, but not progesterone, to liver homogenates obtained from adrenalectomized rats produced a dose-related inhibition of prolactin binding (29).

The *in vitro* effect of dexamethasone on CRF binding to liver and prostate gland appears to be specific, since progesterone, a well-known glucocorticoid antagonist, did not significantly alter CRF binding. The observed dexamethasone inhibition of CRF binding could be mediated by glucocorticoid receptors because whole-liver or -prostate homogenate is required. Dexamethasone is unable to exert an inhibitory effect in the membrane preparations which is devoid of glucocorticoid receptors.

The physiological significance of dexamethasone-inhibited CRF binding *in vitro* is presently unclear; rather high concentrations of dexamethasone are needed for significant inhibition. One possible explanation is that our studies were carried out at 22 C, and it may be that lower concentrations of the steroid would be effective at or near physiological temperatures. Unfortunately, incubation of hepatic and prostatic homogenates to 37 C for 10 min, without dexamethasone, resulted in more than an 80% loss of CRF receptors (Dave & Eskay, unpublished observations); therefore, this hypothesis could not be reliably tested. Alternatively, the homogenates may be partially inactivating the steroid, thus requiring higher amounts of the steroid to produce an effect. In any event, our observations may be of considerable clinical interest, since pharmacological doses of glucocorticoids are given to patients in certain pathophysiological conditions.

REFERENCES

1. Spiess, J., Rivier, J., Rivier, C. and Vale, W. (1981) Proc. Natl. Acad. Sci. USA. 78, 6512-6521.
2. Vale, W., Spiess, J., Rivier, C. and Rivier, J. (1981) Science. 213, 1394-1397.
3. Turkelson, C.M., Arimura, A., Culler, M.D., Fishback, J.B., Groot, K., Konda, M., Licino, M., Thomas, C.R., Chang, D., Chang, J.K. and Shimizu,M. (1981) Peptides. 2, 425-429.
4. Al-Noaemi, M.C., Edwardson, J.A. and Hughes, D. (1982) J. Physiol. (Lond) 332, 85-86.
5. Proulx-Ferland, L., Labrie, F., Dumont, D., Cote, J., Coy, D.M. and Sueirab, J. (1982) Science. 217, 62-63.
6. Sutton, R.E., Koob, G.F., Le Moal, M., Rivier, J. and Vale, W. (1982) Nature. 297, 331-333.
7. Levine, A.S., Rogers, B., Kneip, J., Grace, M. and Morley, J.E. (1983) Neuropharmacology. 22, 337-339.
8. Ehlers, C.L., Hinriksen, S.J., Wang, S.J., Rivier, J., Vale, W. and Bloom, E.F. (1983) Brain Res. 278, 332-338.
9. Valentino, R.J., Foote, S.L. and Aston-Jones, G. (1983) Brain Res. 270, 363-367.
10. Fischman, A.J. and Moldow, R.L. (1982). Peptides. 3, 149-153.
11. Olschowka, J.A., O'Donohue, T.L., Mueller, G.P. and Jacobowitz, D.M. (1982) Peptides. 3, 995-1015.
12. Cote, J., Lefevre, G., Labrie, F. and Barden, N. (1983) Regul. Peptides. 5, 189-195.
13. Palkovits, M., Brownstein, M.J. and Vale, W. (1983)Neuroendocrinology. 37, 302-305.
14. Upton, G.V. and Amatruda, Jr T.T. (1971) N. Engl. J. Med. 285, 419-421.
15. Suda, T., Demura, H., Demura, R., Wakabayashi, I., Nomura, K., Odagiri, E. and Schizume, K. (1977) J. Clin. Endocrinol. Metab. 44, 440-448.
16. Nieuwenhuyzen Kruseman, A.C., Linton, E.A., Lowry, P.J., Rees, L.H. and Besser, G.M. (1982) Lancet. 2, 1245-1246.
17. Petrusz, P., Merchenthaler, I., Maderdrut, J.L., Vigh, S. and Schally, S. (1983) Proc. Natl. Acad. Sci. USA. 80, 1721-1725.
18 Suda, T., Tomori, N., Tozawa, F., Demura, H., Shizume, K., Mouri, T., Miura, Y. and Sasano, N. (1984) J. Clin. Endocrinol. Metab. 58, 919-924.
19. Wynn, P.G., Aguilera, G., Morell, J. and Catt, K.J. (1983) Biochem. Biophys. Res. Commun. 110, 602-608.
20. Desouza, E.B., Perrin, M.H., Rivier, J., Vale, W. and Kuhar, M.J. (1984) Brain Res. 296, 202-207.
21. Dave, J.R., Eiden, L.E., Karanian, J.W. and Eskay, R.L. (1986) Endocrinology, 118 (in press).
22. Dave, J.R., Eiden, L.E. and Eskay, R.L. (1985) Endocrinology, 116, 2152-2159.
23. Dave, J.R. and Eskay, R.L. (1986) J. Clin. Invest. (Communicated).
24. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-273.
25. Thorell, J.I. and Johansson, B.G. (1971) Biochim. Biophys. Acta. 251, 363-369.
26. Dave, J.R. and Witorsch, R.J. (1985) Am. J. Physiol. 248, E687-E693.
27. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-673.
28. Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220-226.
29. Dave, J.R., Witorsch, R.J. and Kalimi, M.Y. (1985) Biochim. Biophys. Acta. 845, 276-282.