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Corticotropin Releasing Factor and Its Binding Protein

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LOWRY, P. J., R. J. WOODS AND S. BAIGENT. Corticotropin releasing factor and its binding protein. PHARMACOL BIOCHEM BEHAV 54(1) 305–308, 1996.—Although the lack of ACTH releasing activity of the high peripheral plasma levels of corticotropin releasing factor (CRF) of human placental origin can now be accounted for by the action of a specific sequestering plasma binding protein (pBP), there are many regions of the brain where the BP is found with little or no overlap with CRF. The existence of a mechanism promoting the rapid disappearance of pBP following bolus injection of exogenous CRF into normal individuals, which is triggered by the formation of a dimer complex (BP2/CRF2), and the elevation of pBP levels found in inflammatory disease, coupled with the lack of unequivocal evidence for endogenous CRF in many of these situations, suggests a role for pBP interaction with ligands other than CRF. We have searched for novel BP ligands in the brain and periphery and have found evidence for them in extracts of sheep brain and in synovial fluid collected from the joints of arthritic patients. These novel BP ligands could, thus, be the peptides responsible for many of the roles currently assigned to brain, peripheral, or immune CRF.

Corticotropin releasing factor Binding protein Ligand Pregnancy Arthritis Inflammation

CORTICOTROPIN releasing factor (CRF), a 41-residue peptide (43), was originally identified in the hypothalamus and was named as such because of its ability to stimulate the release of ACTH from the anterior pituitary gland. The distribution of CRF, however, is not restricted to the brain, as it has been found in many tissues throughout the body (23). One of the surprisingly concentrated sources of CRF is the human placenta, where both the peptide and its mRNA can be detected in significant amounts (16,38). This results in large concentrations of CRF appearing in the maternal blood during the third trimester of pregnancy, reaching levels just before parturition that rival those found in the hypothalamic portal blood during stress in experimental animals (6). Despite this, ACTH concentrations during this period remain within the normal physiological range (35). The discovery (27), purification (2), and subsequent cloning and expression of a plasma protein (33), which can completely abolish the ACTH releasing activity of CRF because of its high affinity and specific binding characteristics, has at least explained the apparent lack of the ACTH releasing activity of CRF in plasma during pregnancy (25). As the binding protein (BP) levels begin to fall during the last few weeks of pregnancy, the equivalence point (i.e., when [CRF] = [BP]) being reached at approximately 3 weeks before parturition whether labor is premature or late (30), it is attractive to postulate that the increasing release of biologically available free CRF then participates in (or initiates), at this crucial time, the events leading to parturition. If the role of the BP, also shown to be produced in the placenta (32), was simply to inhibit the ACTH releasing activity of CRF from placental tissue, then the modulatory action of plasma BP could be assumed to be one purely involved with the events surrounding pregnancy. Its synthesis by the liver (33), which secretes it into the plasma of males and nonpregnant individuals, and its reduction in concentration when exogenous synthetic hCRF is infused would indicate otherwise (46). The expression of the BP in many parts of the brain, including the cortex where no CRF has been found (34), and the failure to detect CRF in the peripheral plasma of nonpregnant individuals (26) would suggest a situation that did not involve CRF but a related peptide. A further indication of a potential physiological function of the BP is corroborated by the observation that following a bolus injection of human CRF, sufficient to stimulate the pituitary adrenal axis, plasma concentrations of the binding protein fall within a few minutes reaching a nadir within 15 min. This appears to be independent of the HPA axis, as ovine CRF is without effect on plasma BP levels, a property that seems to be linked with its low affinity for the human binding protein. We have interpreted that this rapid disappearance of the BP from plasma is triggered by association with ligand, in this case hCRF, and

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have postulated a specific uptake mechanism at site(s) unknown at present (46). We have also shown that when CRF binds to the binding protein the complex forms a stable dimer, a conformational change that can be detected by circular dichroism (28), and have concluded that this change could be the signal that causes specific uptake (47). More recently, we have shown that temporary denaturation with urea leads to a second stable conformational state of the binding protein that neither binds ligand nor crossreacts appreciably with the antiserum against the active form. We can catalyze the reactivation of this inactive BP isoform by incubation in the presence of excess antibody raised against the active form (21). If this isoform phenonemon is not restricted to in vitro experimentation but occurs in vivo, the BP/CRF dimer receptor uptake/activation mechanism, dissociation of the complex, and denaturation of the binding protein followed by reactivation of the latter could represent a novel and interesting mechanism by which the BP and its ligand interact with a specific receptor followed by possible discharge of the ligand.

We have for some time now been monitoring a number of nonpregnant clinical situations and, apart from liver and kidney failure, the only patients in which we have found the levels of BP to be different from normal are those suffering from arthritis and septicaemia. Whereas in late pregnancy the levels of BP were lower than normal, in both arthritis and septicaemia the concentrations were significantly elevated [(13); Fig. 1]. This could be one explanation for the defective hypothalamic response of patients with rheumatoid arthritis to immune inflammatory stimuli (9) due to the increased sequestering capacity of plasma BP in the hypothalamic portal system. Whereas the lowering of BP in pregnancy appears to be triggered by secretion of placental CRF, the increased levels found in these active immune states appears to be caused by increased liver secretion, presumably due to activation of enhancer elements implicated in the acute phase response, which can be found in the 5′ flanking region of the BP gene (3). One at −305 is known to bind the transcription factor NF-kB known to regulate immunoglobulins and interleukins, and is thought to be important in activating liver-specific genes such as angiotensinogen in response to inflammation or injury (36). Another element was located at −676, which binds INF-1, a transcription factor known to regulate the interferon gene (14). The common link between these conditions is the immune system, pregnancy being a condition in which immune suppression is important, and arthritis and septicaemia, in which the immune system is overactive. Indeed, it is well known that rheumatoid arthritics can go into remission during pregnancy only to flare up again after parturition (18). Unfortunately, the function of the known ligand for BP, i.e., hCRF, in the immune system is somewhat controversial. The underlying theme from observations has been that CRF or immune CRF of peripheral origin is involved in the inflammatory cascade, appearing to play a role in the initiation and propagation of the inflammatory response in concert with other local factors. CRF receptors have been found on human peripheral blood lymphocytes and monocytes (39) and mouse spleen macrophages (44). CRF has direct effects in vitro on cells of the immune system including stimulation of lymphocyte proliferation and expression of the interleukin-2 receptor (41,29) and stimulation of secretion of interleukin-1 and interleukin-2 from leucocytes (40). Systemic immunoneutralization of endogenous CRF-like peptides has been shown to cause suppression of chemically induced aseptic inflammation in normal rats (20), and expression of CRF has been demonstrated in the joints of adjuvant-induced arthritis in Lewis rats (12). More recently, the latter group have detected CRF in the synovial fluids and tissues of patients with rheumatoid arthritis (11). Conversely, centrally administered CRF has been shown to suppress the immune response by attenuating the humoral response to antigen (19). Given all this evidence it is difficult to believe that the role of peripheral or immune CRF has not become widely accepted. Indeed, we have been cautious in this respect, as on a number of occasions we have exposed inherent artefacts prevalent in the use of antibodies in radioimmunoassay and immunocytochemistry (5,15,23) and have some experience with the problems using PCR to detect mRNA.

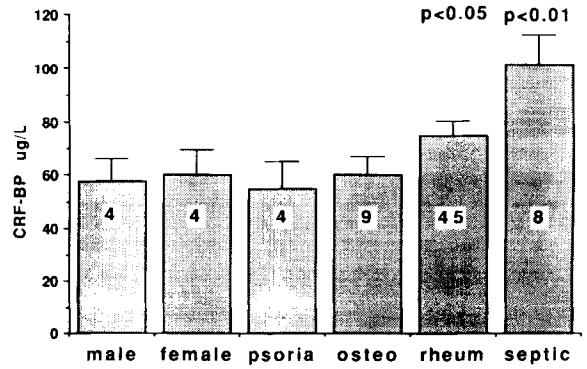

FIG. 1. CRF-binding protein concentrations in plasma samples taken from patients suffering with psoriatic, osteo- or rheumatoid arthritis and septicaemia. The error bars represent ± SEM, and statistical significance was calculated with respect to combined data from the male and female controls.

Other observations that have encouraged us to predict and search for this CRF-related peptide are:

1. The α-helical CRF antagonist can inhibit the acute phase response to interleukin 1β, whereas immunoneutralization of systemic CRF with an antibody (which can sequest the ACTH releasing activity of synthetic CRF at the pituitary) was without effect (17).
2. The α-helical antagonist is a more effective antagonist of CRF at peripheral vasoactive receptors than at pituitary receptors (10) and is a potent ligand for human CRF-BP (42), the association with which is accompanied by a unique change in conformation as detected by circular dichroism (28).
3. As well as hypothalamic CRF, fish have a further CRF-related peptide found in the urohypophysis, urotensin 1, which has a 10-fold higher affinity for the human CRF-BP than hCRF (42).
4. Ovine CRF has a 15-fold less affinity than hCRF for sheep brain BP (4).
5. Recently, there have been a number of receptors that have been cloned that have higher affinities for sauvagine and urotensin 1 than h/rCRF, with a peripheral receptor displaying a spectrum of activity almost identical to that of the human binding protein (UT1 > Sauv > hCRF ≫ oCRF) (8,31,7,22).

This growing body of evidence has led to our reappraisal especially with our prediction and recent discovery of a novel CRF-related peptide (1), which could not be detected with our specific two-site IRMAs for oCRF and hCRF, but has a high affinity for the human binding protein. This indicates that it

shares common structural domain(s) with hCRH that may be the same epitopes on this new peptide that are responsible for the crossreaction seen with some CRF antibodies in studies that lack uneqivocal evidence for CRF.

For our strategy at Reading to isolate the BP ligand we elected to use the ovine brain as our starting material, as due to its low affinity for BP, oCRF would not interfere in the competitive hBP ligand binding assay used to monitor the purification of the putative peptide. The assay is performed by preincubation of 2.5 ng of recombinant hBP with standard hCRF or unknown followed by a further incubation with [^{125}I]-hCRF. Separation is achieved by incubation with an excess of a sheep anti-BP antibody followed by a precipitating donkey antisheep antibody. We also used specific, separate, IRMAs for oCRF (37) and hCRF (24) during the purification as a check of integrity of purified fractions. Sheep brains were homogenized in 3 vol (v/w) of methanol (containing 1 mM NEM/PMSF) and the supernatant rotary evaporated to one-third of its volume. Extraction on Sep-Pak resulted in both ligand and oCRF eluting in the 50/60% acetonitrile step-wise fractions. Preparative HPLC of these fractions separated the new ligand from the oCRF peak, the ligand being slightly more hydrophobic (Fig. 2). A minute peak of hCRF immunoreactivity was detected in the oCRF position. This material was then submitted to affinity chromatography on solid phase BP and resulted in separation of oCRF from the new ligand, the latter predictably being more tightly bound to the BP solid phase. A final analytical HPLC step resulted in a peptide that was essentially pure, as judged by *N*-terminal squence analysis and electrospray mass spectrometry. The latter also assigned it a unique mass expected for a peptide of some 41 residues. This new sheep brain peptide also appeared to have a higher affinity than hCRF for hCRFBP. These properties indicate that this is a unique mammalian peptide that could be a potential ligand for some of the newly recognised CRF receptors.

In view of the occurrence of BP in the blood and, in particular, its increased concentration in inflammatory disease, we have reexamined synovial fluid from patients with arthritis for CRF-like material. Because of its thick consistency synovial fluid is difficult to handle. Nevertheless, we have been able to achieve a peptide-rich fraction that is amenable to concentration on Sep-Pak. Step-wise elution resulted in BP ligand and hCRF being detectable in the 30/40/50% acetonitrile fractions with at least 10-fold more BP ligand than could be accounted for by the hCRF immunoreactivity. Separation on HPLC resulted in the separation of the bulk of ligand appearing in earlier fractions than the small peak of hCRF detected in the hCRF immunoradiometric assay. This early elution of ligand when compared with CRF is due either to the human ligand being less hydrophobic or its partial degradation before or during extraction (45).

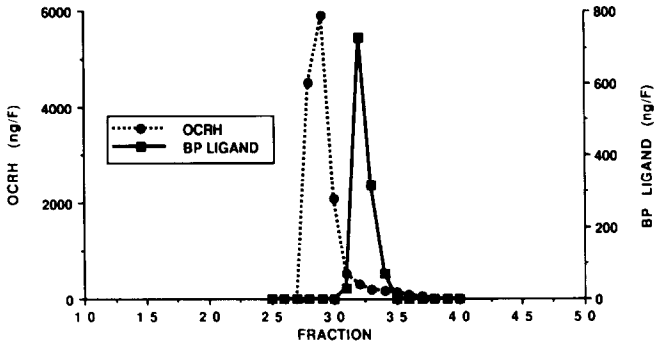

FIG. 2. Semipreparative HPLC of ovine brain extract on Hi-Pore RP318 (250 × 10 mm) column. Extract was derived from five ovine brains and eluted with an acetonitrile gradient (20–70%) in 0.2% TFA. Flow rate was 3 ml/min with 1.5 ml fractions collected. Fractions were assayed for BP ligand using a BP ligand assay (see text) and for oCRF using a CRF specific two-site IRMA in which primary antibodies were unlabeled rabbit anti-CRF 1-20 and iodinated sheep anti-CRF 21-41.

In conclusion, there would appear to be other peptide(s) related to CRF in the brain and periphery that could be potential ligands for the CRFBP and the newly cloned receptors. These peptides may play and explain many of the extra hypothalamic roles assigned to CRF, which until recently were the subject of much controversy, as many of the conclusions were reached in the absence of any real evidence for either ligand or receptor. It seems now we have candidates for both.

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