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Research report

Corticotropin-releasing factor (CRF) can directly affect brain microvessel endothelial cells

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Abstract

Stress activates the hypothalamic–pituitary–adrenal (HPA) axis through release of corticotropin releasing factor (CRF), leading to production of glucocorticoids that down regulate immune responses. However, acute stress via CRF also has pro-inflammatory effects. We previously showed that acute stress increases rat blood–brain barrier (BBB) permeability, an effect involving brain mast cells and CRF, as it was absent in W/W^v mast cell-deficient mice and was blocked by the CRF-receptor antagonist, Antalarmin. We investigated if CRF could also have a direct action on brain microvessel endothelial cells (BMEC) isolated from rat and bovine brain. BMEC were cultured and identified by electron microscopy. Western blot analysis of cultured BMEC identified CRF receptor protein; stimulation with CRF, or it structural analogue urocortin (Ucn) showed that the receptor is functionally coupled to adenylate cyclase as it increased cyclic AMP (cAMP) levels by 2-fold. These findings suggest that CRF could affect BMEC structure or function, as reported for increased cAMP levels by other studies. It is, therefore, possible that CRF may directly regulate BBB permeability, in addition to any effect mediated via brain mast cells.

Theme: Cellular and molecular biology

Topic: Blood–brain barrier

Keywords: BBB; cAMP; CRF; Endothelial cells; Stress; Multiple sclerosis

1. Introduction

The BBB is made up of layers of brain endothelial cells [19], astroglia, pericytes, and perivascular mast cells surrounded by basal lamina. Brain microvessel endothelial cells (BMEC) are characterized by tight junctions that restrict passage of most molecules into the brain. The protective function of the BBB can be altered during various disease states of the central nervous system (CNS), specifically during cerebral inflammation [9]. BBB permeability has been shown to increase in response to stress [2,31,32,34].

Stress activates the hypothalamic–pituitary–adrenal (HPA) axis through the release of CRF, leading to secretion of catecholamines and glucocorticoids; these, in turn, downregulate the immune response [6]. CRF is synthesized predominantly in the paraventricular nucleus (PVN) and mediates its effects through at least three types of receptors (R): CRFR-1, CRFR-2α and CRFR-2β. However, both CRF and these receptors are present throughout

Abbreviations: BBB, blood–brain barrier; cAMP, cyclic AMP; CNS, central nervous system; CRF, corticotropin-releasing factor; HPA, hypothalamic–pituitary–adrenal; MS, multiple sclerosis; TNF-α, tumor necrosis factor-α

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the neuraxis indicating that CRF, or structurally related compounds such as urocortin (Ucn) [40], might also have paracrine actions.

CRF also has proinflammatory effects [20], apparently mediated in part through mast cell activation [39]. For instance, acute restraint stress was shown to induce intracranial rat mast cell degranulation, an action that was CRF-dependent [38]. As mast cells secrete many vasoactive mediators, we hypothesized they could regulate the BBB [37]. This proposal was supported by findings that the mast cells secretagogue, compound 48/80, increased BBB permeability in the mast cell rich habenula of pigeons [43]. We recently reported that acute restraint stress increases BBB permeability in rats, an effect apparently mediated through CRF activation of diencephalic mast cells [11]. These results suggest that CRF secreted from the hypothalamus or elsewhere in the brain could regulate the BBB through brain mast cells.

Conversely, CRF was recently shown to decrease BBB permeability in a rat model of cerebral ischemia [17]. Moreover, some studies indicated that elevation of cAMP induced the formation of tight junctions in cultured brain endothelial cells [30,41]. Nevertheless, the possibility that CRF may also have a direct effect on brain endothelial cells has not been studied. In order to investigate this possibility, we isolated and cultured rat and bovine brain endothelial cells. Western blot analysis detected CRFR protein and stimulation with CRF or Ucn showed that the CRFR is functionally coupled to cAMP. These findings suggest that CRF may have direct regulatory actions on brain microvessels.

2. Materials and methods

2.1. Isolation of BMEC from rat and bovine brain

Primary cultures of BMEC were prepared as previously described [1] and characterized with light and electron microscopy. Cerebral cortices, or other brain regions as noted, were removed from either rat or bovine brain; they were finely minced at room temperature and then incubated in Dispase solution (0.8% Dispase in MEM-F12 containing antibiotics) for 2 h at 37 °C in a shaking waterbath. The pellet containing the microvessels was washed once in DMEM-F12 and was separated on a 30% dextran gradient. The pellet was digested for another 2.5 h at 37 °C with collagenase-dispase solution (1 mg/ml in MEM-F12 containing antibiotics). The cell suspension was carefully layered on a continuous 50% Percoll gradient and centrifuged at $1000\times g$ for 10 min at room temperature. The band of endothelial cell clusters was aspirated, washed twice in MEM-F12 and then plated onto rat-tail collagen-coated 60-mm^2 plastic dishes (Fisher Scientific, Springfield, NJ). The BMEC became confluent on the fifth day of culture.

2.2. Light and electron microscopy

The exclusive presence of endothelial cells was confirmed using light and electron microscopy as described before [38].

2.3. Detection of CRFR on BMEC by Western blot analysis

Cells were harvested from the growth dishes, were immediately lysed and were prepared for Western blot analysis. Samples were run on a 15% SDS–PAGE gel for 1 h and were then transferred to an Immobilon membrane (BioRad, Hercules, CA). The membrane was incubated with goat anti-CRFR-1 that recognized both CRFR-1 and CRFR-2 (CRFR-1, Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1:1000 for 1 h at room temperature. Following washing, the membrane was incubated with rabbit anti-goat HRP-conjugated serum (Santa Cruz) at a dilution of 1:10 000 for 30 min at room temperature. The protein band was detected using ECL chemoluminescence (BioRad).

2.4. cAMP assay

CRF (1 μM) was added to the cell cultures that were further incubated for 5, 15 and 30 min at 37 °C. Control cells were incubated with medium only. Following incubation time, cells were lysed directly on the growth dish using the detergent provided by the cAMP enzyme immunoassay kit (RPN225, Amersham–Pharmacia, Piscataway, NJ). Following trypan blue staining to ensure complete lysis, the cell lysate was collected and assayed for cAMP. In some cases, the BMEC were treated with forskolin or pretreated either with the CRFR antagonist Antalarmin (1 μM, kindly donated by Dr. G. Chrousos, NIH) or the ATP analogue 2′5′-deoxyadenosine for 5 min at 37 °C.

2.5. Statistics

The cAMP data were analyzed using a one-sample Student's *t*-test, which is commonly used to compare one experimental group to baseline, which is taken as zero. Significance is denoted by $P<0.05$.

3. Results

3.1. Characterization of BMEC

BMEC were prepared from whole rat or bovine brain, as well as from individual brain regions. The results described below were obtained with BMEC from rat brain. Under light microscopy, cultured BMEC exhibited a characteristic 'cobblestone-like' appearance (Fig. 1A).

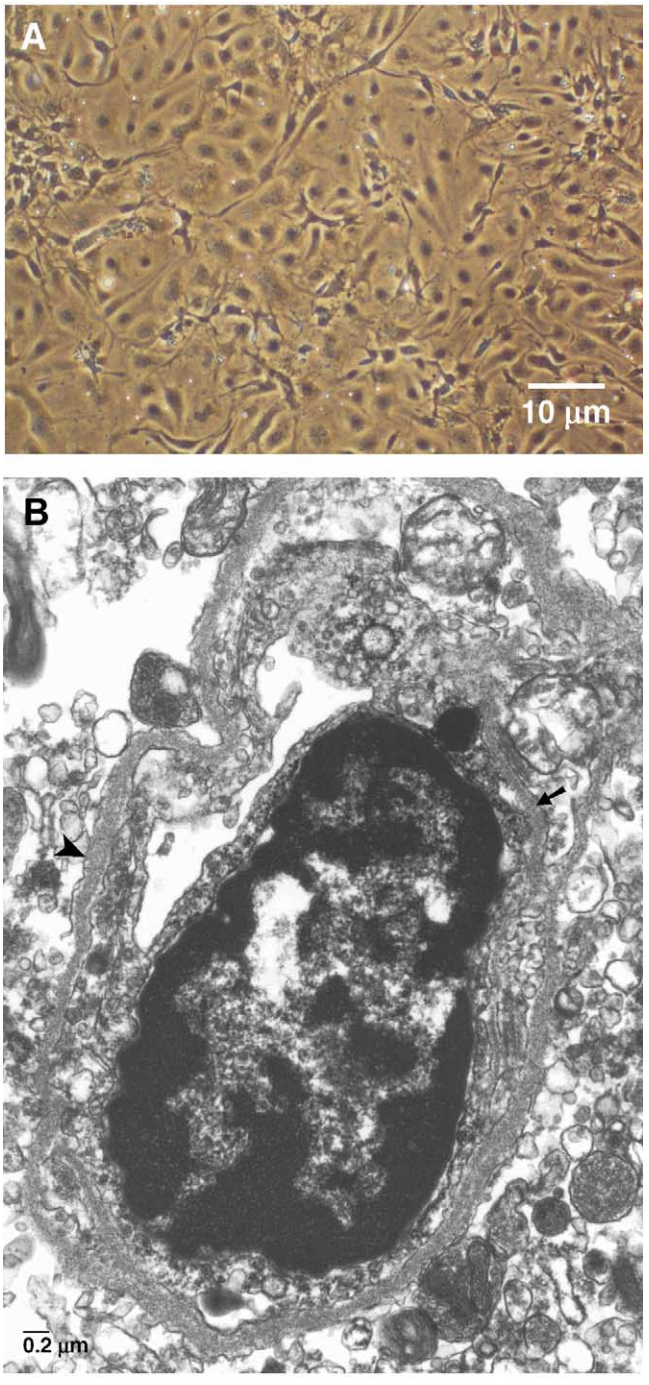

Fig. 1. (A) Photomicrographs of BMEC: (A) adherent BMEC not stained; scale bar=10 μm. (B) One detached BMEC examined by transmission electron microscope (arrowhead, basement membrane; arrow, endothelial cell membrane); scale bar=0.2 μm.

Electron microscopy confirmed the identity and exclusive presence of endothelial cells, as there were no other contaminating cells (Fig. 1B).

3.2. Western blot analysis of CRFR in BMEC

Western blots using antibodies to both CRFR on pituitary samples (which express large amounts of CRFR-1) and cerebellar BMEC yielded a similar single band of about 49 kDa (Fig. 2A). Western blot analysis detected a similar single band of 49 kDa (Fig. 2) in BMEC isolated from all brain regions (Fig. 2B). Based on the amino acid sequence, the two known forms of the CRF receptor are expected to have a molecular weight between 46 and 53 kDa. As the available anti-CRFR antibody used recognizes both receptor types, it is not possible to determine at this time which subtype is present.

3.3. Effect of CRF on BMEC cAMP levels

CRF has been shown to exert its effects through binding to CRF receptors and activation of adenylate cyclase. In order to investigate if the CRFR on BMEC is functional, cAMP levels were measured following treatment with CRF or Ucn. Stimulation of BMEC with CRF (100 nM) increased cAMP significantly ($P<0.05$) by 1.6±0.1-fold above baseline at 5 min, with a maximum of 2.0±0.1-fold above basal at 30 min (Fig. 3A). The intracellular cAMP levels decreased slowly after this time, but still remained above basal values at 1 h. Similar findings were obtained when Ucn (100 nM) was used, instead; however, in this case, equivalent maximal increase was achieved at 15 min, instead of the 30 min required for CRH (Fig. 3B). The effect of CRH was compared to forskolin-induced cAMP production which at 30 min was 4.53±0.65-fold higher than basal in BMEC; pretreatment of cells with Antalarmin (1 μM for 5 min at 37 °C) reduced the effect of CRF on cAMP to below basal levels at all time points (results not shown). The effect of CRF was also blocked by pretreatment with the ATP analogue 2′5′-deoxyadenosine (1 μM for 5 min at 37 °C).

4. Discussion

This is the first time that CRF receptor protein has been shown to be expressed on BMEC, but the receptor subtype present could not be identified. The commercially available antibodies used for both CRFR-1 and CRFR-2 evidently recognized both receptor types. Based on the amino acid sequence of both receptors, the predicted molecular weights range between 46 and 53 kDa. As the two peptides have very similar molecular weights, it is possible that Western blotting cannot distinguish between the two, despite the different specificities of the antibodies used. Because these peptides are often glycosylated, some investigators report the receptors to be of larger molecular weight, anywhere from 49 to 80 kDa [5,18]. It could also be possible that the receptor subtype on brain microvessels may be a different splice variant recognized by both antibodies. In addition to the well established CRFR-1, CRFR-2α and CRFR-2β, there have been reports of other variants that may be involved [27].

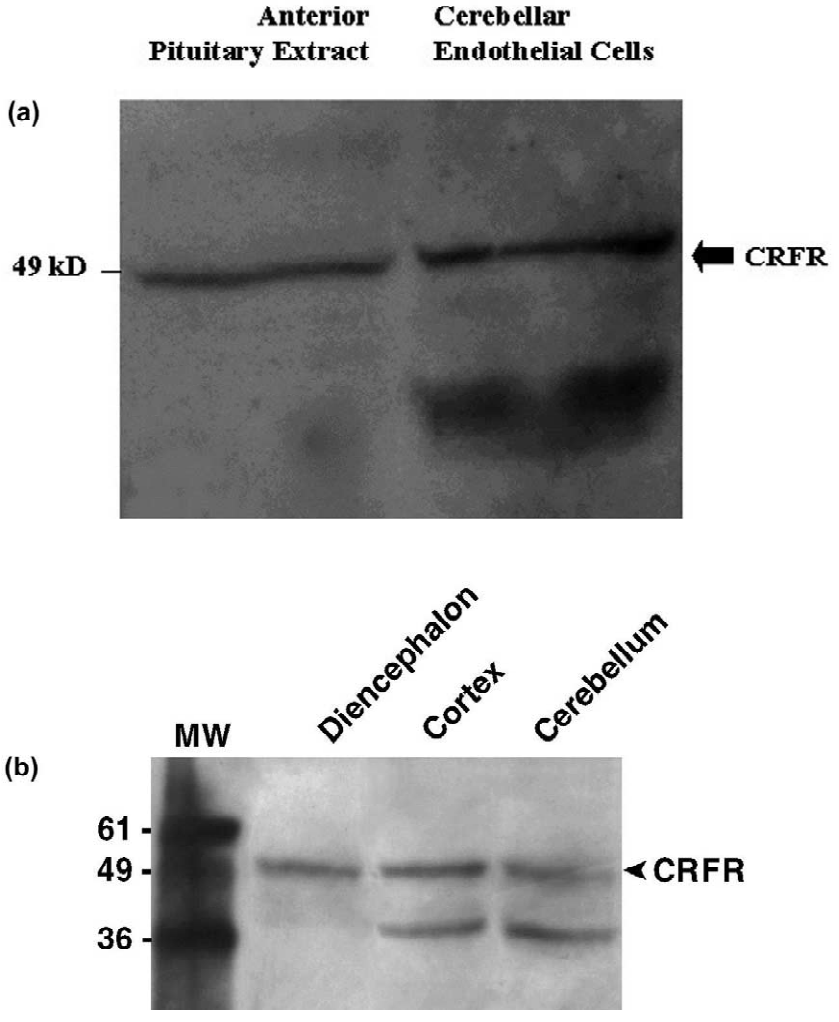

Fig. 2. Western blot analysis of the presence of CRFR in BMEC of various brain regions as indicated (n=3). The arrow points to the CRFR identified by the antiserum directed towards CRHR-1, but evidently also recognizing CRHR-2; the lower MW band shown in two lanes is nonspecific. (A) Pituitary extract is compared to cerebellar BMEC; (B) cerebellar BMEC extract is compared to those from cortex and diencephalon.

There have been a number of reports that acute stress increases BBB permeability in animals [2,31,32,34] and in humans [15]. We had previously shown that acute stress induces BBB permeability to [^{99}Tc]gluceptate through CRF and mast cells [11]. This finding was confirmed with site injection of CRH in the hypothalamus and absence of any effect of acute stress on BBB permeability in W/W^{v} mast cell deficient mice [12]. These results, along with the finding that mast cells also express CRFR [36,39], indicate that CRF may affect BBB permeability through mast cell activation, especially in the diencephalon, as well as by a direct action on endothelial cells elsewhere in the brain. Mast cells have been reported to be associated with microvascular endothelial cells [29], but their presence in BMEC cultures was excluded with the use of electron microscopy.

A direct action of CRF on BMEC is supported by the fact that CRF binds to peripheral endothelial cells and inhibits their synthesis of prostaglandins [13]; moreover, CRF has been shown to induce vasodilation in the human fetal–placental circulation [7]. Moreover, cultured human umbilical vein endothelial cells have been shown to secrete CRF and to express CRFR-2 receptor mRNA [33].

Our studies also demonstrate that the CRFR present on BMEC is functional as it led to cAMP elevations in response to CRF or Ucn, in the range of what was achieved by forskolin used as a positive control. The single concentration of 100 nM used was chosen because (a) this concentration is attainable in the diencephalon, assuming 100–500 ng/g CRF and a molecular weight of 4757 for rat and human CRF; (b) this concentration was reported to result in maximal cAMP elevations in mouse anterior pituitary fetal rat extrahypothalamic forebrain cell cultures {Kapcala, 1995} cells {11894}, and (c) because we recently showed that site injection of 1 mM CRF, assuming a 10-fold dilution in the hypothalamus, resulted in measur-

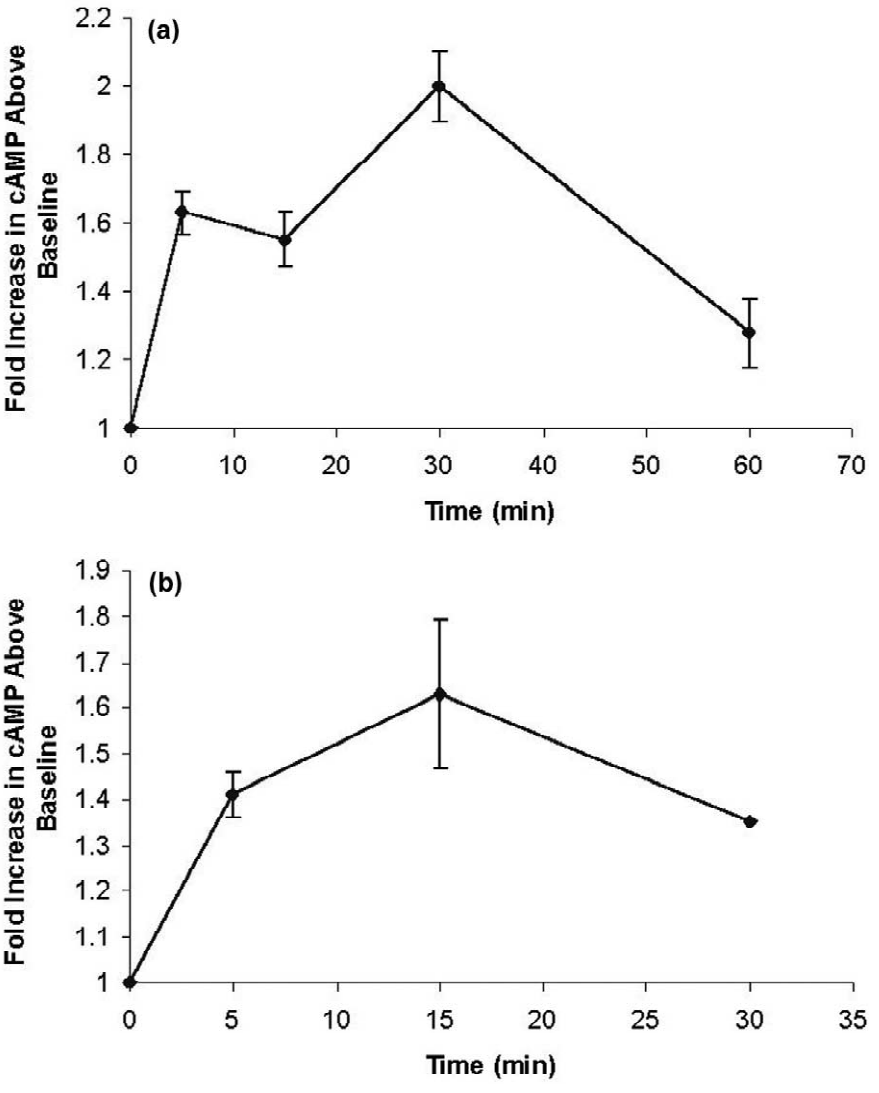

Fig. 3. Effect of (A) CRH or (B) Ucn (100 nM) on cAMP levels in BMEC ($n=3$). The results at all points were statistically significant, when compared to control treated with PBS.

able increase in BBB permeability in the rat [12]. It is possible that the 2-fold induction of cAMP we detected is low because of a low affinity CRF receptor or damage to CRF receptor during the purification process. However, a 2-fold camp increase may be sufficient to initiate the signal cascade leading to morphological changes [42]. Previous studies had shown that elevations in cAMP induced the formation of tight junctions in cultured brain endothelial cells [30,41]. Alternatively, cAMP may initiate the mitogen-activated protein (MAP) kinase pathway that results in the activation of NFκB, ultimately leading to transcription of pro-inflammatory genes, such as those for cytokines [23]. There is evidence that NFκB activity is increased in endothelial cells during inflammation [23] and that CRF affects NFκB in neurons [24]. Preliminary attempts to investigate whether CRF could affect NFκB activity in BMEC were unable to detect any activation because BMEC exhibited very high basal activation of NFκB (results not shown).

Acute stress was also shown to shorten the time to onset of EAE in mice [4]. Moreover, breakdown of BBB integrity has been documented to precede any clinical symptoms or pathological findings in multiple sclerosis (MS) [21] and symptoms in relapsing-remitting MS often appear to worsen by psychological stress [14,26]. BBB permeability increase may occur through CRH acting on brain mast cells [10–12] and CRF was shown to be involved in traumatic brain injury [35]. This premise is supported by reports that the mast cell secretagogue compound 48/80 increased BBB permeability in pigeons [43]. Moreover, local application of 48/80 to pia induced BBB permeability to fluorescein-labeled dextran [25], while histamine typically released from mast cells increased BBB permeability to [^{99m}Tc]sodium pertechnetate

or [^{131}I]serum albumin [3]. TNF-α could also be involved since it is released along with histamine from rat hypothalamic mast cells [8], has been shown to regulate BBB permeability [22] and is increased in the cerebrospinal fluid (CSF) of MS patients [16].

The direct effect of CRF or Ucn on BMEC may occur either (a) at different times, or (b) in different parts of the brain than its effect on brain mast cells and could have the opposite effect on BBB permeability than the increase induced through mast cells. This possibility is supported by the recent report that intracisternal administration of CRF decreased BBB permeability induced by cerebral ischemia [17]. However, this effect was more pronounced during the chronic phase implying that it may depend on HPA axis activation. CRF administration also reduced the development of experimental allergic encephalomyelitis [28], possibly through a combination of HPA axis activation and a direct effect on T-cells, as it also suppressed EAE in adrenalectomized rats.

The present results extend our understanding of the effect of CRF on brain endothelial cells and, possibly, on BBB permeability and indicate that there may two opposing direct and indirect actions may have opposite effects.

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