NF-κB Participates in the Corticotropin-releasing, Hormone-induced Regulation of the Pituitary Proopiomelanocortin Gene*

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Katia P. Karalis‡, Maria Venihaki§, Jie Zhao§, Lilian E. van Vlerken, and Christina Chandras
From the Division of Endocrinology, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Corticotropin-releasing hormone is a main regulator of mammalian stress response by stimulating pituitary proopiomelanocortin (POMC) gene expression, and thus adrenocorticotropin hormone (ACTH) secretion, which then causes glucocorticoid release from the adrenal. In a recent study in the pituitary corticotroph cell line AtT20, oxidative stress stimulated the activity of nuclear transcription factor B (NF-κB), whereas corticotropin-releasing hormone (CRH) inhibited both the constitutive and the oxidative stress-induced NF-κB DNA-binding activity. To further investigate the role of NF-κB on the CRH-induced pituitary POMC gene activation, AtT20 cells were transiently transfected with a POMC-luciferase construct mutated at an NF-κB binding site. After treatment with CRH, intracellular POMC-luciferase activity was significantly higher from the stimulation observed with transfection of the parental POMC-luciferase construct. In agreement with a previous report, CRH inhibited the constitutive NF-κB DNA-binding activity in AtT20 cells, as shown by electrophoretic mobility-shift assay, as soon as within 15 min of treatment. These effects of CRH were blocked by the CRH-R1 antagonist CP154,256. Our findings provide evidence that the regulation of corticotropin NF-κB activity by CRH is related to the activation of the pituitary POMC gene and, thus, may play an important role in stress response.

Hypothalamic corticotropin-releasing hormone (CRH) acts as a major mediator of the mammalian stress response by stimulating pituitary proopiomelanocortin (POMC) gene expression and adrenocorticotropin hormone (ACTH) secretion that, in turn, stimulates release of glucocorticoid from the adrenal gland (1). CRH stimulates POMC gene transcription in pituitary corticotrophs through cAMP and calcium-mediated events (2). Positive and negative regulation of the POMC gene has been described and shown to be mediated by several transcription factors such as AP-1 (3), Nur77 (4), Ptx1 (5), and glucocorticoid receptor (6).

The Crh-deficient (Crh−/−) mouse has normal basal circula-
RNA Extraction and Northern Blot Hybridization—AtT20 cells were plated in six-well tissue culture dishes and allowed to adhere for 24 h. Cells were transiently transfected with a plasmid containing the full-length cDNA of human l-BSa, subcloned in Invitrogen PCDNA3 vector at the EcoRI site, kindly provided by Dr. Simeonides, Beth Israel Deaconess Medical Center, Boston, MA. Twenty hours later, both transfected and non-transfected cells were treated with either vehicle (saline) or 10^{-7} M CRH. Cells were isolated 18 h later and RNA was extracted, as we have previously reported, by using Trizol reagent (Sigma). RNA (10 μg) was separated on a 1.4% formaldehyde agarose gel and transferred to GeneScreen (PerkinElmer Life Sciences) following standard protocols (12). A complementary RNA POMC riboprobe was labeled with α-32PdUTP (PerkinElmer Life Sciences) and T7 polymerase, as previously described (13). Hybridization was carried out at 65°C for at least 16 h with 10^6 cpm riboprobe/lane. The filter was washed (3 × 20 min in 0.1× SSC-1% SDS) and exposed to Kodak XAR 5 film at room temperature for 30 min.

Isolation of Nuclear Extracts—AtT20 cells were harvested, and the cell pellets were lysed in ice-cold hypotonic lysis buffer containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM NaF, 1 mM NaVO4, and 1% Nonidet P-40 for 10 min. After a brief centrifugation at 3000 × g for 1 min, the cytosolic extracts were collected while the nuclear pellets were lysed in high-salt extraction buffer containing 20 mM HEPES-KOH (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl2, 0.3 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 0.1% Triton X-100, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin. After incubation on ice for 45 min with intermittent vortexing, the nuclear extracts were collected, followed by centrifugation at 14,000 × g for 30 min at 4°C, and their protein concentration was determined with the BCA protein assay kit (Pierce) using bovine serum albumin as a standard. The nuclear extracts were stored at −80°C until further use.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from AtT20 cells were subjected to EMSA analysis. Double-stranded oligonucleotides of the core sequence of the NF-κB binding element on mouse immunoglobulin κ light chain (sense, 5′-TCG, ACA GAG GGG ACT TTC TCG, 3′; antisense, 5′-TCG, AGC CTC TGG AAG, AGT CCC CTC TG-3′) were labeled with [32P]dCTP (50 Ci/mmol, Amersham Biosciences) and 2 μl of the 32P-labeled probe for 30 min at room temperature. For the supershift studies, 2 μl of antibody (anti-p65, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture and incubated for another 20 min at room temperature before the addition of the 32P-labeled probe. The DNA-protein-binding complexes were analyzed by EMSA on a nondenaturing 6% polyacrylamide gel using a Tris/glycine/EDTA buffer. After being dried, the gel was exposed to film at −70°C.

Statistical Analysis—All experiments were performed at least three times. Data were analyzed by Student’s t test and one-way analysis of variance followed by post-hoc multiple comparison tests. Significance was accepted at p < 0.05.

RESULTS

CRH Inhibits Pituitary NF-κB—We evaluated the effect of CRH on pituitary NF-κB DNA-binding activity using AtT20 cells, a pituitary corticotroph cell line. Our findings showed inhibition of the NF-κB DNA-binding activity by CRH (Fig. 1A), which is in agreement with a previous report (10), whereas lipopolysaccharide (LPS), an inducer of the NF-κB DNA-binding activity in immune cells, had a similar effect in AtT20 cells as well (Fig. 1A). The effect of CRH was apparent within 15 min of treatment and lasted at least 6 h after its addition to the culture (Fig. 1B). This effect of CRH was blocked by CP154,526, a nonpeptide-specific CRHR1 antagonist (Fig. 2). Challenge of the cells with dexamethasone inhibited the NF-κB DNA-binding activity in immune cells, had a similar effect in AtT20 cells as well (Fig. 1A). The effect of CRH was apparent within 15 min of treatment and lasted at least 6 h after its addition to the culture (Fig. 1B). This effect of CRH was blocked by CP154,526, a nonpeptide-specific CRHR1 antagonist (Fig. 2). Challenge of the cells with dexamethasone inhibited the NF-κB DNA-binding activity, analogous to the effect of dexamethasone on the expression of NF-κB in immune cells (Fig. 2). Finally, co-addition of CRH and dexamethasone resulted in a greater inhibition of the NF-κB DNA-binding activity than each agent alone (Fig. 2), suggesting that these hormones might activate this transcription factor by means of independent mechanisms.
NF-H9260B-dependent Effect of CRH on Pituitary POMC Gene Expression—The importance of pituitary NF-H9260B on the CRH-induced regulation of the POMC gene expression was studied by transfecting AtT20 cells with a plasmid containing the POMC promoter coupled to the luciferase reporter gene either intact or mutated at an NF-H9260B binding site. Cells were treated with 10^{-7} M of CRH for 6 h or vehicle (control). The effect of CRH on POMC gene expression was significantly enhanced in the cells transfected with the mutated plasmid. *, p < 0.05, n = 4 wells/experiment.

NF-H9260B-dependent Effect of CRH on Pituitary POMC Gene Expression—The importance of pituitary NF-H9260B on the CRH-induced regulation of the POMC gene expression was studied by transfecting AtT20 cells with a plasmid containing the POMC promoter coupled to the luciferase reporter gene either intact or mutated at an NF-H9260B binding site. Cells were treated with 10^{-7} M of CRH for 6 h or vehicle (control). As has been shown previously (11), CRH treatment of AtT20 cells transfected with the intact construct resulted in 2.5–3× induction of the transcriptional activity of the POMC gene (Fig. 3A). CRH treatment of the AtT20 cells transfected with the mutated construct resulted in a further increase (6× over the control) of the transcriptional activation of the POMC gene (Fig. 3A). To elucidate the pathway mediating the above effects of CRH, we pretreated AtT20 cells transfected with either the WT or the mutant construct for 1 h with 10 μM of H89, PKA, or GF109203X, the PKC inhibitor, before the 6-h treatment with 10^{-7} M CRH. H89 abolished the CRH-induced transcrip-
of POMC mRNA was significantly increased (2.5×) after CRH treatment in the cells overexpressing LxBo. These data suggest that induction of NF-κB DNA-binding activity is an important pathway for the CRH-induced stimulation of the pituitary POMC gene expression.

DISCUSSION

In mammals, activation of the hypothalamic-pituitary-adrenal (HPA) axis constitutes the main endocrine response to stress and is mediated primarily by increased expression of the hypothalamic CRH (14). By binding to CRHR1 on pituitary corticotrophs, CRH stimulates pituitary POMC transcription and thus secretion of ACTH, which leads to glucocorticoid release (15). CRH-induced POMC expression is mediated through the cAMP/protein kinase A pathway and through calcium-dependent events (2). Other regulators of POMC such as leukemia inhibitory factor have been found to act independently of cAMP and may work by stimulation of the transcription factor STAT3 (16–18).

NF-κB is a transcription factor that regulates the expression of a variety of proinflammatory genes. NF-κB DNA-binding activity is induced by proinflammatory factors as well as by cellular stresses such as heat and hypoxia (19). NF-κB is expressed in the brain; a recent study (10) showed that the hypoxia-induced NF-κB DNA-binding activity in hippocampal neurons is inhibited by CRH, suggesting protective effects of CRH on brain cells suffering ischemia-induced damage. This finding provided a mechanism for the suggested neuroprotective effects of CRH. In this report, we demonstrate that induction of pituitary NF-κB DNA-binding activity participates in the transcriptional regulation of the POMC gene by CRH, a critical step of the stress response. In a study evaluating the effect of heating stress on NF-κB activation in a lung cell line, there was inhibition of the NF-κB DNA-binding activity in lung cells that paralleled the degree of stimulation of heat-shock proteins (20). These findings suggested that the inhibition of the NF-κB DNA-binding activity may be part of the tissue-specific stress responses.

In previous studies (21), we showed that CRH stimulates the NF-κB DNA-binding activity in immune cells. This effect of CRH is attributed to the proinflammatory action of peripheral CRH, and it is unlikely to be mediated by the CRH receptor 1 because a CRHRR1-specific antagonist did not block this effect of CRH. On the other hand, the effect of CRH on pituitary NF-κB should be mediated by CRHR1, the only shown CRH receptor subtype expressed in corticotroph cells. The above suggests receptor-specific effects of CRH on the regulation of transcription factors such as NF-κB, and, furthermore, they raise the hypothesis of differential regulation of NF-κB by immune versus physical/psychological stressors.

Our findings suggest that the inhibitory effect of CRH on the NF-κB DNA-binding activity in pituitary cells is related to the transcriptional activation of the POMC gene by CRH (Fig. 4). Activation of the POMC gene leads to ACTH secretion, a step necessary for the release of glucocorticoid, the end product of the activated HPA axis. Three putative regulatory elements on POMC promoter have been found to bind CRH-induced transcription factors. The AP-1 site in exon 1 (+41/+47) binds CRH-induced cAMP-response element-binding protein, cFos, and junB. Nurr1 and Nur77 bind to two specific sequences: −70∥−63, which corresponds to a pivotal responsive sequence for positive or negative POMC regulation by Nurr and the glucocorticoid receptor, respectively, or −404∥−383, that recognizes Nur77 homodimers or heterodimers of the POMC promoter (4, 22). We found stimulation of the CRH-mediated transcriptional activation of POMC after mutation of a traditional NF-κB binding sequence. There is no overlap of this site with any of the above well-characterized sequences on the POMC promoter. In addition, there is no overlap between this sequence and the suggested glucocorticoid-responsive elements in the POMC promoter. Negative regulation of the POMC gene by glucocorticoids by means of antagonism between the glucocorticoid receptor and factors such as the orphan nuclear receptor Nur77 or the AP-1 has been shown (4, 23, 24), but to our knowledge, inhibition of the transcriptional stimulation of POMC gene by any other transcription factor has not been reported.

In summary, inhibition of NF-κB DNA-binding activity is associated with the transcriptional activation of the POMC gene. It has been well shown that transcriptional activation of the POMC gene is associated with activation of the AP-1 factor (25). Parallel activation of NF-κB and AP-1, which leads to increased transcription, has been described for several genes related mainly to activation of the immune system (26). Our findings suggest opposing roles for NF-κB and AP-1 in the activation of the POMC gene in corticotroph cells. The contribution of NF-κB in the regulation of the induced expression of other pituitary hormones and its physiological significance remains to be determined.

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