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Dexamethasone-induced up-regulation of the human norepinephrine transporter involves the glucocorticoid receptor and increased binding of C/EBP-β to the proximal promoter of norepinephrine transporter

Qinqin Zha, **Yan Wang**, **Yan Fan**, and **Meng-Yang Zhu**
Department of Pharmacology, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA

Abstract

Previously, we have found glucocorticoids upregulate norepinephrine transporter (NET) expression *in vitro*. However, the underlying transcriptional mechanism is poorly understood. In the present study, the role of glucocorticoids on the transcriptional regulation of NET was investigated. Exposure of neuroblastoma SK-N-BE(2)M17 cells to dexamethasone significantly increased NET mRNA and protein levels in a time- and dose-dependent manner. This effect was attenuated by glucocorticoid receptor antagonist mifepristone, suggesting that upregulation of NET by dexamethasone was mediated by the glucocorticoid receptor. In reporter gene assays, exposure of cells to dexamethasone resulted in dose-dependent increases of luciferase activity that were also prevented by mifepristone. Serial deletions of the NET promoter delineated dexamethasone-responsiveness to a −301 to −148 bp region containing a CCAAT/enhancer binding protein-β (C/EBP-β) response element. Coimmunoprecipitation experiments demonstrated that dexamethasone treatment caused the interaction of the glucocorticoid receptor with C/EBP-β. Chromatin immunoprecipitation (ChIP) assay revealed that dexamethasone exposure resulted in binding of both glucocorticoid receptor and C/EBP-β to the NET promoter. Further experiments showed that mutation of the C/EBP-β response element abrogated C/EBP-β- and glucocorticoid receptor-mediated transactivation of NET. These findings demonstrate that dexamethasone-induced increase in NET expression is mediated by the glucocorticoid receptor via a nonconventional transcriptional mechanism involving interaction of C/EBP-β with a C/EBP-β response element.

Keywords

Dexamethasone; gene expression; norepinephrine transporter; glucocorticoid receptor; transcription; protein-protein interaction

Introduction

Stress is one of the physiological challenges that can alter brain functions. During stress, enhanced activity of the hypothalamic-pituitary-adrenal (HPA) axis results in an elevated level of circulating glucocorticoids. While homeostasis of glucocorticoids within the normal physiological range regulates the level of arousal and influences vital signs, chronic stress

Correspondence to: Dr. Meng-Yang Zhu, Department of Pharmacology, Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37604, Phone: (423) 439-6394, Fax: (423) 439-8773, zhum@etsu.edu.

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and subsequent excessive glucocorticoid secretion endanger neuronal structural integrity and function. As a result, the exaggerated HPA activity produced by chronic stress has been associated with the development of depressive symptoms in humans. Additionally, the noradrenergic system serves as an integral part of the stress response system in an organism, helping to regulate arousal and adapt to environmental and physiological stressors (Robbins and Everitt 1995). Dysfunction of noradrenergic transmission is also thought to be causally involved in the development of depression (Charney 1998). Since both chronic stress and dysfunctional noradrenergic transmission are implicated as etiological factors of depression, the molecular interaction between them may be the basis for the depressed state. Stress-released glucocorticoids (cortisol in humans and corticosterone in rodents) are the final effector of stress-activated HPA and play essential roles for adaptive stress responses. Therefore, these interactions most likely occur between glucocorticoids and the phenotype of the noradrenergic systems. Likely, elucidating the molecular biological interaction between them *in vivo* and *in vitro* will uncover the molecular mechanism underlying their contribution to the pathophysiology of depression.

The norephnephrine (NE) transporter (NET) is a protein located on presynaptic terminals of noradrenergic neurons in the brains and peripheral nervous systems (Iversen 1971). NET has been recognized as a hallmark of these neurons owing to its restrictive expression in noradrenergic neurons (Barker and Blakely 1995). In synaptic clefs of noradrenergic neurons more than 90% of released NE are transported back to presynaptic terminals by NET (Axelrod and Kopin 1969), a primary mechanisms for inactivation of NE transmission. As such, alterations of NET expression would remarkably affect NE levels in the synapses and in turn highly influence noradrenergic transmission. Our previous study demonstrated that treatment of SK-N-BE(2)M17 and PC 12 cells with stress-relevant doses of corticosterone increased mRNA and protein levels of NET, as well as the uptake of [^{3}H]NE (Sun et al. 2010). Although the mediation role of corticosteroid receptors in corticosterone-induced upregulation of NET expression and function has been recognized, the involved molecular biological mechanism needs to be elucidated. Clarification of these molecular mechanisms could facilitate the search for effective therapies for major depression and other stress-related diseases.

Many molecular mechanisms are involved in the gene regulation. Several studies have shown that in cells treated with glucocorticoids, the activation at the transcriptional level is a primary mechanism for increased expression of target genes (Eberwine et al., 1984; 1987; Karin, 1998). Likewise, both transcriptional and post-transcriptional controls affect gene expression, because they determine the cellular concentration of mRNA and therefore the capacity for protein synthesis (Belasco, 1993; Grunberg-Manago, 1999). Usually, the transcriptional responses are mediated by transcription factors that interact with cognate *cis*-acting DNA sequences within the promoter region of target genes. Clarifying these important factors and structures is an important step to explore the molecular mechanism underlying glucocorticoid-induced expressional upregulation. In this study, we have used neuroblastoma cells to investigate which transcription factors mediate dexamethasone (Dex)-caused induction of NET genes as the first step. The results showed that the interaction of the glucocorticoid receptor (GR) and CCAAT enhance binding protein-β (C/EBP-β) is involved in this regulation, which may lead us to further explore other important structures involved in this regulation.

Material and Methods

Cell culture and drug exposure

The human neuroblastoma SK-N-BE(2)M17 cell line (by courtesy of Dr. K.S. Kim, Molecular Neurobiology Laboratory, McLean Hospital, Harvard Medical School) was

maintained as described previously (Zhu et al. 2002; Sun et al. 2010). Culture medium and supplements were obtained from Gibco-Invitrogen (Carlsbad, CA). Drug exposures in experiments were started after 3 days of each subculture, once cells had become confluent. Dex (Sigma, St Louis, MO) was dissolved in 40 μl dimethylsufoxide and then further diluted with saline. The same amount of vehicle was added into the drug-free medium for cells in the control group. When cells were treated with Dex, FBS was replaced by dialyzed FBS. Dex (0, 1, 10, 50, 100, 1000 n*M*), alone or in combination with mifepristone or actinomycin D (both from Sigma, St Louis, MO), was added into 6-well plates in fresh media. Cells were harvested after exposing to these chemicals at the time indicated in the text. Microscopic examination of cells for possible toxic effects of these compounds was conducted routinely in cultures, as described previously (Zhu et al. 2002).

Reporter gene and deletion constructs

A parental promoterless and enhancerless luciferase reporter vector, pGL3-basic is gifted from Dr. Zhihua Han, using which several NET promoter-luciferase reporter constructs were prepared. The upstream primers containing KpnI site and the downstream primer bearing the HindIII site were designed as follows: pGLhNET-F1, 5' CCT GGT ACC GCT GGG TTA ATG CAA TCG3'; pGLhNET-F2, 5'GCC GGT ACC GTG ACG TTA AGT GTC CG 3'; pGLhNET-F3, 5'GCG GTA CCA AAC GAG GAA AAG TGC TGC3'; pGLhNET-F4, 5' CTT GGT ACC GCT CAT CCC AGT GTC TAA G 3'; antisense primer RPhNET, 5' ATT AAG CTT TGG ATG CGG CTG GC 3'. These primers were then synthesized by Integrated DNA Technology (Coralville, IA). The locations of the primers in the putative NET promoter are illustrated in Fig. 6A. The primer set (pGLhNET-F1/RPhNET) was used to isolate the DNA sequence from −777 to +1 region (the "A" nucleotide of the translation start codon ATG is designated as +1) through PCR using human genomic DNA as templates. The human genomic DNA was prepared from 2×10^7 SK-N-BE(2)M17 cells in a 150-mm culture dish in triplicates. Aliquots of the DNA solution were used for cloning according to standard molecular cloning methods. Restriction digestion and direct sequencing were used to screen and confirm clones, respectively. The 778 base pair (bp) full-length NET reporter construct was denoted as pGLhNET-F1. Using three other designed primer sets, three different deletion constructs with a DNA insert of 435-, 301-, and 148-bp 5'-upstream from the translation start codon ATG were obtained, respectively. These deletion construct vectors were assigned as pGLhNET-F2, pGLhNET-F3, and pGLhNET-F4 (Fig. 6A).

Transfection and luciferase reporter assay

For transfection, SK-N-BE(2)M17 cells were plated in 24-well plates at a density of 2×10^4 per well 24 hr before transfection. Cells were transfected with different promoter-constructs, using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer's manual. Two ng Renilla reniformis luciferase plasmid pRL-TK (Promega, Madison, WI) was included for normalization. Dex was added 24 hr after transfection. Forty-eight hr after transfection, cells were washed with phosphate-buffered saline (PBS) and harvested by 1x passive lysis buffer (Promega, Madison, WI). The firefly and Renilla reniformis luciferase activities were determined by a luminometer (Turner Designs, Oak Ridge, TN) using Dual-Luciferase Reporter Assay systems (Promega, Madison, WI) according to the manufacturer's protocol.

Site-directed mutagenesis

Within the pGLhNET-F1 construct, the C/EBP-β response element (TGCTGCAAG, −288 to −280) in the proximal promoter region was mutated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotide primers were designed to anneal to the same sequence on the opposite strand of the plasmid flanking the C/EBP-β response

element and carrying a two-base pair AA-TT mutation. The primer sequences are as follows: sense 5'-GAGGAAAAG**TGCTGCTTG**GTCTTCAGCCGCCC-3' and antisense 5'-GGGCGGCTGAAGACC**AAGCAGCA**CTTTTCCTC-3', in which the response element is indicated by bold letters, and the mutated bases are underlined. The presence of the mutation was confirmed by direct sequencing and resultant plasmid was designated as pGLhΔNET–F1.

RNA Isolation, semiquantitative RT-PCR and quantitative real-time polymerase chain reaction (qPCR) analysis

Isolation of total RNA and semiquantitative RT-PCR were performed as described previously (Sun et al., 2010). Bands were visualized using G Box (Scion Corporation, Frederick, MD, USA), where appropriate semiquantitative analysis of RT-PCR signals was carried out by Genetools software (SynGene, Cambridge, England) and the intensities of β-actin bands were used for normalization of NET.

Real-time quantitative PCR was performed with an Icycler (Bio-Rad, Hercules, CA) using the SYBR green Platinum Quantitative PCR supermix (Invitrogen, Carlsbad, CA). The following primer sequences were used: NET-F 5'-CGG TGC CTT CTT GAT CCC G-3' and R-5' CCG GTT GTA CTG TCC CAGAG-3'. Human β-2 microglobulin gene was used as a control gene (F-5'-GAC TTT GTC ACA GCC CAA G-3', R-5'-GCA AGC AAG CAG AAT TTG G-3') to normalize for variations in the amount of starting material in each PCR reaction. The primer sequences were designed to span intron–exon boundaries to avoid amplification of genomic DNA. The size of PCR products was: NET, 91 bp and β-2-microglobin, 112 bp. The PCR program was started with 95°C for 3 min, followed by 50 cycles of 95°C for 18 s, and 60°C for 45 s, then continued with the melting curve analysis (55–90°C) to verify the product specificity. To create a standard curve for each gene of interest, human cDNA corresponding to the analyzed region was synthesized by IDT technology (Coralville, IA). A standard curve then was generated by analysis of the serial dilutions of oligo fragment solutions (10^2–10^7 copies/μl). For each sample the copy number of both NET and β-2-microglobulin housekeeping gene was extrapolated from their respective standard curves. The value of NET mRNA expression was normalized with the β-2-microglobulin copy number and expressed in arbitrary units. Reproducibility of results was determined by performing triplicate measurements of each cDNA aliquot.

Western blot analysis and coimmunoprecipitation

Whole cell extracts for Western blot analysis were prepared by lysing cells in ice-cold Nonidet P-40 (NP-40) buffer (0.5% NP-40, 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 5 m*M* EDTA) for 15 min, after which cell debris were removed by centrifugation at 14,000 rpm for 10 min at 4°C. An equal volume of 2x SDS gel-loading buffer was then added to the supernatant and the samples were denatured at 80°C for 5 min. Protein concentrations in cell extracts were quantified spectrophotometrically prior to addition of the loading buffer with the Bio-Rad protein assay kit, according to the instruction of the manufacturer (Bio-Rad, Hercules, CA). Proteins (40 μg) from each whole cell extract were electrophoretically separated on a 10% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Amersham Life Sciences, UK). For protein detection, the immunoblots were probed with a mouse IgG monoclone anti-NET antibody (1:1000, Mab technology Inc., Stone Mountain, GA), or anti-GR (1:5000), anti-C/EBP-β antibody (1:5000) (Santa Cruz Biotechnology Inc, Santa Cruz, CA). A horseradish-peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:5000, Amersham Life Sciences, UK) was used as the secondary antibody. The membranes were then subjected to enhanced chemiluminescence (Amersham Life Sciences, UK) and autoradiography, according to instructions of the manufacturer. To

check for equal loading and transfer, the membranes were reprobed with a mouse IgG monoclonal anti-β-actin antibody (1:3000, Amersham Life Sciences, UK).

Coimmunoprecipitation was performed with a nuclear extraction kit (Active Motif, Calsbad, CA). The nuclear extracts containing 200 μg of proteins were incubated overnight at 4°C with 2 μg of anti-C/EBP-β antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 500 μl of NP-40 lysis buffer. Protein A/G beads (60 μl, Thermo Scientific, Rockford, IL) were added to the mixture and incubated for an additional hour with rocking. The immunocomplexes were then washed 6 times with NP-40 lysis buffer. The immunoprecipitated proteins were dissolved in 60 μl of 2x Laemmli buffer and boiled for 5 min before analysis by Western blotting.

Chromatin immunoprecipitation assay (ChIP)

ChIP was carried out with an EZ-Magna ChIP™ A kit according to the manufacturer's instruction (Millipore Biotechnology, Billerica, MA). Four aliquots of 2×10^7 nuclei were resuspended in 1 ml of RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS at pH 7.4) and chromatins were fragmented to 200–500 bp with 6 pulses of 10 sec at 40% power with a Sonic Dismembrator Model 100 (Fisher Scientific, Pittsburgh, PA) at 4°C. To remove insoluble components, samples were centrifuged at 13,000 rpm for 15 min and supernatants recovered. Rabbit polyclonal antibodies anti-GR or anti C/EBP-β (both from Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG were conjugated to goat anti-rabbit IgG magnetic beads. For each aliquot of 2×10^7 nuclei, 50 μl of magnetic bead slurry were in turn added by 5 μg of primary antibody and the sheared chromatin in RIPA, and then incubated on a rotator overnight at 4°C according to the manufacturer's recommendation.

The DNA in immunoprecipitates was analyzed by PCR using the primer pair of NET F-5'-GCG GTA CCA AAC GAG GAA AAG TGC TGC-3' and R-5'-ATT AAG CTT TGG ATG CGG CTG GC-3' that recognize the −301 to +1 bp region of the NET promoter. The PCR products were separated by agarose gel electrophoresis for detection by ethidium bromide staining.

Statistics

All experimental data are presented in the text and graph as the mean ± SEM, which was obtained from the separate experiments. The number of replicates is described in the legend of figures. Data were analyzed using unpaired Student's *t* test for data in Fig. 8A or one-way analysis of variance (ANOVA) for the rest, which was followed by a *post hoc* Newman-Keuls test for planned comparisons.

Result

1. Dexamethasone induces NET gene expression

In the present study, synthesized glucocorticoid Dex was selected for the characteristics of its relatively high activity, longer half-life than corticosterone, and preference for the GR (Sapolsky 1992). To characterize NET induction by Dex *in vitro*, the dose and time courses following Dex treatment were performed using Western blotting. SK-N-BE(2)M17 cells were exposed to Dex at a concentration of 1, 10, 50, 100, 1000 nM for 24 hr. The selection of these concentrations of Dex is based on the literature and there is no cell toxicity under this condition (Wakade et al. 1996; Son et al. 2001; Golde et al. 2003). This experiment showed a concentration-dependent enhancement of NET protein levels by Dex ($F_{5,17}$=8.79, p<0.01, Fig. 1A). *Post hoc* tests revealed that NET protein levels in cells treated with 10, 100, and 1000 nM Dex were significantly increased by 1.9- (p<0.05), 3.7- (p<0.01), and 6.9-

fold (p<0.01, Fig 1A), respectively. Time course study for NET proteins showed a marked increase of NET at 4 hr after 10 nM Dex treatment, and NET protein levels remained elevated for at least 24 hr (Fig. 1B).

Next, NET mRNA levels in SK-N-BE(2)M17 cells treated with different concentrations of Dex for 8 hr or 10 nM Dex for 24 hr were analyzed by quantitative real-time PCR. Fig. 2 shows a concentration- and time-dependent induction of NET mRNA, a pattern similar to Dex-induced increase in NET protein levels. Ten nM Dex treatment for 24 hr resulted in a 3.3-fold increase in NET mRNA, as compared to the control group (p<0.01).

To examine whether Dex induces transcriptional activation of the NET gene, two approaches were applied: using the pharmacological inhibitor and the NET promoter reporter gene assay. First, SK-N-BE(2)M17 cells were pretreated with 0.5 μM actinomycin D, an inhibitor of RNA synthesis, for 30 min before exposing to 10 nM Dex for 8 hr. Results showed that while actinomycin D treatment alone had no effect on NET expression, it prevented Dex-induced increases in NET mRNA (Fig. 3A) and protein levels (Fig. 3B). Second, as mentioned in the Material and Methods, a chimeric luciferase gene fused to the 778 bp part of the human NET promoter (pGLhNET-F1) was constructed, based on our pre-experimental screening after cloning of the NET promoter region. This construct was transiently transfected into SK-N-BE(2)M17 cells and maintained in serum-supplemented media for 24 hr. These transfected cells were then exposed to vehicle or different concentrations of Dex for 24 hr. As shown in Fig. 4, a significant increase of reporter gene activity was initially observed at a concentration of 1 nM Dex with a 2.5-fold increase in luciferase activity, which was followed by a higher increase resulting from the treatment with 10 nM Dex. However, this 10 nM Dex-induced 3.9-fold increase looked like the maximal effect, as further increases in reporter activity were not observed when 100 and 1000 nM Dex were applied. These data indicate that Dex indeed causes transcriptional activation of the NET gene.

2. Dex-induced induction of NET occurred via the GR

Glucocorticoids are known to diffuse through the membrane to enter cells, where they bind to corticosteroid receptors in the cytosol. These hormone-receptor complexes are then translocated to the nucleus to affect transcription. To examine the mediation role of corticosteroid receptors in the upregulation of NET expression by Dex, SK-N-BE(2)M17 cells were exposed to 10 nM Dex, alone or in combination with 1 μM mifepristone (RU 486), a GR antagonist. Western blotting results showed that co-treatment with mifepristone prevented Dex from inducing NET protein expression (Fig. 5A). The involvement of the GR in the transcription was also investigated. SK-N-BE(2)M17 cells were transfected with NET reporter gene construct pGLhNET-F1, maintained in serum-supplemented medium for 24 hr and the cells were respectively treated with 10 nM Dex, 1 μM mifepristone, or a combination of both drugs for another 24 hr. As shown in Fig. 5B, Dex treatment was associated with a significant increase in luciferase activity as compared with vehicle-treated cells. While treatment with mifepristone alone had no significant effect on luciferase activity, this antagonist blocked the increase in luciferase activity caused by Dex. These data demonstrated that activation of GR was necessary for Dex-induced transcription of the NET gene.

3. Dex-induced increases in NET transcriptional activity are mediated through the C/EBP-β response element

To explore possible mechanisms underlying Dex-induced upregulation of NET expression through transcriptional activation, a promoter sequence search was performed for a potential glucocorticoid-responsive elements (GREs), a structure for binding of glucocorticoid to

constitute a glucocorticoid response unit to activate the transcription (Karin, 1998; Schoneveld et al. 2004), within 800 bp upstream of the translational start site, based on the reporter gene assay result presented in Fig. 4. However, it failed to find this consensus sequence within this region of the NET gene promoter. Next, sequential deletion from the 5' end of the NET promoter within this 800 bp region was carried out to identify the potential regulatory elements. The reporter genes containing progressive deletions of 5'-flanking region of the NET promoter were constructed. Transfections were then performed to determine the response of these four reporter gene constructs to activation of NET by 10 nM Dex. It appears that on three constructs including that of 5' deletion upstream of −301 bp Dex treatment significantly activated NET promoter activity. In contrast, the −148 bp NET promoter construct (pGLhNET-F4) did not show significant activation on NET promoter by Dex (Fig. 6A). These data indicate that between −149 and −777 bp of the NET promoter, some elements are responsible for Dex-mediated transactivation of the NET gene. In an effort to delineate the possible location, a specific *cis*-element site in the NET promoter was identified. Between −301 bp and −148 bp of the NET promoter, a C/EBP-β response element stands out as a well-defined *cis*-element (Fig. 6B), which is known to be bound by C/EBP-β, a transcription factor from C/EBP family (Boruk et al. 1998).

4. GR and C/EBP-β interaction in Dex-induced NET expression

Since glucocorticoids act through glucocorticoid receptors to enhance or repress transcription of glucocorticoid-responsive genes, whether the GR interacts with C/EBP-β during its transactivation of the NET gene was examined. To fulfill this goal, a coimmunopreciptitation experiment was performed in SK-N-BE(2)M17 cells which were exposed to 10 nM Dex, alone or in combination with 1 μM mifepristone for 24 hr. In the immunoprecipitates of C/EBP-β, the GR was detectable in Dex-treated cells but not in vehicle-treated cells. Mifepristone was able to prevent the GR from interacting with C/EBP-β (Fig. 7A). This result indicates the interaction of the GR and C/EBP-β for Dex action. Furthermore, to examine whether Dex treatment induces the GR and C/EBP-β binding to the NET promoter *in vitro*, a chromatin immunoprecipitation (ChIP) assay was carried out. As shown in Fig. 7B, Dex treatment caused increased GR and C/EBP-β to bind to the −301/+1 region of the NET promoter. The binding was abolished by simultaneous treatment of cells with Dex and mifepristone. With normal rabbit IgG, there was no binding of the NET promoter, a demonstration for the specificity of the ChIP method.

5. Dex increased nuclear GR and C/EBP-β protein expression in a time dependent manner

We hypothesized that Dex-mediated transactivation of the NET gene in SK-N-BE(2)M17 cells was due, in part, to the formation of complexes between GR and C/EBP-β. The effect of Dex on GR and C/EBP-β nuclear protein levels was also examined by Western blotting. Treatment of SK-N-BE(2)M17 with 10 nM Dex for 8 hr resulted in an increase in GR proteins in nuclear extracts; however, total GR proteins remained unchanged (Fig. 8A). To get an overall sense about the potential role of C/EBP-β in the regulation of NET expression by Dex, we further examined the expression pattern of C/EBP-β in response to Dex. As shown in Fig. 8B, Western blotting detected a major 45 kDa and a minor 43 kDa band which are different isoforms of C/EBP-β proteins. Dex treatment of SK-N-BE(2)M17 cells led to an increase of the expression of C/EBP-β proteins in a time dependent manner ($F_{4,14} = 4.10$, $p < 0.05$).

To determine whether the C/EBP-β response element plays a critical role in Dex-responsiveness, the C/EBP-β response element within pGL-hNET F1 was mutated by site-directed mutagenesis and luciferase reporter assays were performed after transfection of cells with this mutant construct pGLΔhNET1. Treatment of transfected cells with different

concentrations of Dex did not have any significant effect on luciferase activities (Fig. 9), indicating that the C/EBP-β response element is required for Dex induced NET activation.

Discussion

The present study demonstrates that Dex induces the elevation of NET expression in neuroblastoma cell line SH-N-BE(2)M17. This action of Dex is through activation of the GR, as simultaneous treatment with Dex and mifepristone, a GR antagonist, totally blocked the elevation of NET expression. Mechanistic studies indicated that C/EBP-β, a transcription factor that regulates transcription through CCAAT DNA sequence motifs (Ramji and Foka 2002), appeared to play the essential role in Dex-induced upregulation of NET expression. First, in reporter gene transfection assay, the construct of NET promoter region, which contains the C/EBP-β response element, exhibited Dex responsiveness. Mutating C/EBP-β response element prevented Dex from inducing NET gene expression. Second, protein binding profiles, such as coimmunopreciptitation and ChIP assay, of this *cis*-element were changed by Dex. Third, Dex exposure significantly increases protein levels of C/EBP-β. It seems that by binding to the NET promoter in close proximity to the translational start site, the physical interaction between the GR and C/EBP-β occurred as a result of Dex exposure. These lines of evidence suggest that the interaction between GR and C/EBP-β on the NET promoter triggers transcriptional activation of the NET gene. Present observations are consistent with those in the literature that activated GR interacts with C/EBP-β physically (Boruk et al. 1998; Rudiger et al. 2002), that C/EBP-β mediates effects of glucocorticoids on gene expression (Yamada et al. 1999; Kimura et al. 2001; Sun et al. 2008).

As a main hormone released during stress, glucocorticoids affect cellular and molecular events in brains by modulating the expression of many genes during stress. It was reported that the number of genes per cell directly regulated by glucocorticoids is estimated to be between 10 and 100 (Srinivas et al. 1989). The previous (Sun et al. 2010) and current studies demonstrated that glucocorticoids significantly increase the expression of NET *in vitro*, indicating that a possible transactivation is involved. Generally, glucocorticoids exert their transcriptional regulation through the glucocorticoid-receptor homodimer which binds with high affinity to specific GREs in promoter regions of target genes to constitute a glucocorticoid response unit to activate the transcription (Karin, 1998; Schoneveld et al. 2004). To seek potential GRE sites, we delineated the region (−777 to +1) within the proximal promoter through deletion analysis of the 5’ flanking region of the NET gene. Transient expression of this reporter gene revealed a very strong GR-dependent Dex induction of NET transcription (Fig. 4). However, computer analysis of this region failed to identify GRE equivalents, suggesting that the observed Dex responsiveness was due to a nonconventional glucocorticoid response. It was reported that transcriptional control by glucocorticoids is alternatively mediated by a family of transcription factors that were activated by ligands and bind to *cis* regulatory elements in specific target genes (Scheidereit et al. 1983; Carson-Jurica et al. 1990). Among these transcription factors, C/EBP family is one master regulator of many cellular responses. While members of this family play pivotal roles in cell growth, metabolism, immune and inflammatory processes (Ramji and Foka 2002), C/EBP-β has been shown to be widely expressed in the mammalian nervous system (Lekstrom-Himes and Xanthopoulos 1998; Sterneck and Johnson 1998) and plays a key role in memory and learning (Sterneck et al. 1998; Taubenfeld et al. 2001a; Taubenfeld et al. 2001b), a function also overlapped by the noradrenergic system (Robbins and Everitt 1995). Interestingly, computer analysis shows a C/EBP-β response element within the NET proximal promoter (−301 to −148). The further experiments including site-directed mutagenesis of this response element (Fig. 9) confirm the mediation role of C/EBP-β through the C/EBP-β response element. It is noteworthy that the luciferase activity from construct pGLhNET-F2 is much higher than that of pGLhNET-F1 (Fig. 6A), indicating

there may be an enhancer for Dex-induced NET transcription. More experiments are on the way to indentify this structure and other *cis*-element structures in this region and the rest of the NET promoter.

Many studies have shown that high affinity binding of glucocorticoid receptors to the promoter of target genes is strictly dependent on specific DNA sequences such as GREs (Nishio et al. 1993; Zilliacus et al. 1995). It has been reported, however, that glucocorticoid receptors also participate in chromatin remodeling and interacts with coregulators, resulting in changes in the expression of genes that do not have GREs in the promoter (McEwan et al. 1997; Deroo and Archer 2001; Lonard and O'Malley B 2007). The novelty of the present finding is that the GR and C/EBP-β cooperatively transactivate the NET gene, which is independent of GREs. Nevertheless, although C/EBP-β is most likely required for Dex-mediated induction of NET, the precise relationship between the GR and C/EBP-β in facilitating binding to the C/EBP-β response element is currently unclear. There are several possible mechanisms by which Dex influences C/EBP-β to mediate NET transcriptional activation, such as GR-mediated up-regulation of C/EBP-β expression (Matsuno et al. 1996), increased access to and/or binding of C/EBP-β to the C/EBP-β response element, or direct protein-protein interaction between the GR and C/EBP-β (Nishio et al. 1993). The current study provides some evidence for these potentially underlying mechanisms. For example, Dex exposure significantly increased protein levels of C/EBP-β (Fig. 9). Results of ChIP assay indicated that both C/EBP-β and GR are present at the C/EBP-β response element, suggesting that the GR may perform a tethering role through direct protein-protein interaction. Such a physical interaction has been observed with glucocorticoid receptors and the chicken ovalbumin upstream promoter-transcription factor II that plays a critical role in the metabolism of glucose, cholesterol, and xenobiotics (De Martino et al. 2004). Nevertheless, because the physical interaction occurred through the basic-leucine zipper (bZIP) domain of C/EBP-β, which is highly conserved among its isoforms, it cannot be ruled out that other members of the C/EBP family could also substitute as an accessory factor for this response. Also, it remains to be clarified whether additional transcription factors, or the GRE structures and other *cis*-elements in the rest region of the NET promoter are required for the maximal action of dexamethasone.

In conclusion, we have demonstrated a positive transcriptional control of the NET gene by glucocorticoids, possibly through interactions between the GR and C/EBP-β. DNA sequences upstream from the C/EBP-β response element between −301 and −148 bp in the NET promoter appear necessary for glucocorticoid effects. However, it is understandable that no single factor is responsible for the complex regulation of the NET gene, given many types of machinery are involved in glucocorticoid-induced gene regulation. The present study provides evidence that the transactivation potential of C/EBP-β may be one important way in which the GR exerts physiological effects in the absence of DNA binding. Considering both the noradrenergic system and C/EBP-β are involved in the functions of the memory and learning, further study to elucidate the functional interaction of the GR and C/EBP-β in Dex-induced enhance of NET expression may have potential indication for exploring molecular mechanisms underlying stress and brain functions.

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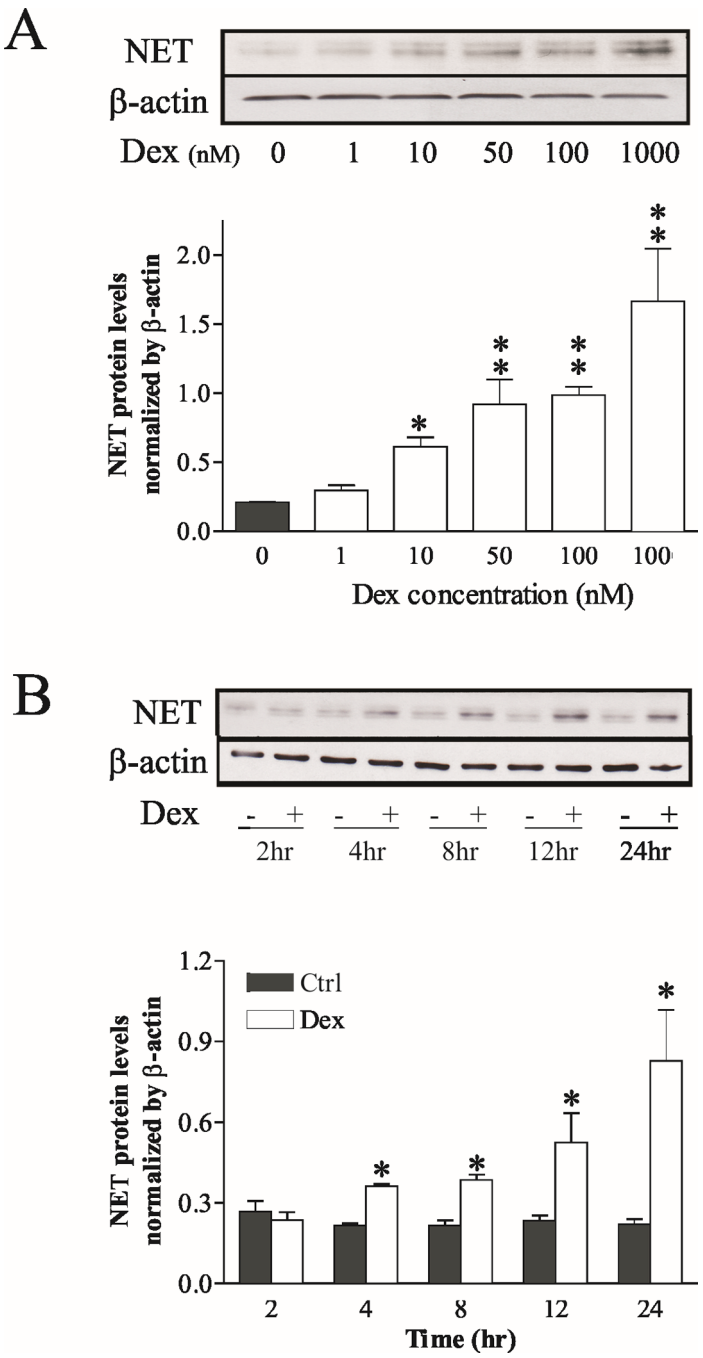


Figure 1.

Effects of Dex exposure on NET protein levels in SK-N-BE(2)M17 cells: concentration (A) and time (B) courses of Dex exposure. The upper panel of each figure shows autoradiograph obtained by Western blotting of NET. The lower panel shows quantitative analysis of band densities. The data represent means ± SEM of three independent experiments. *$p < 0.05$, ** $p < 0.01$; compared to the group treated with vehicle (0) and control groups (Ctrl).

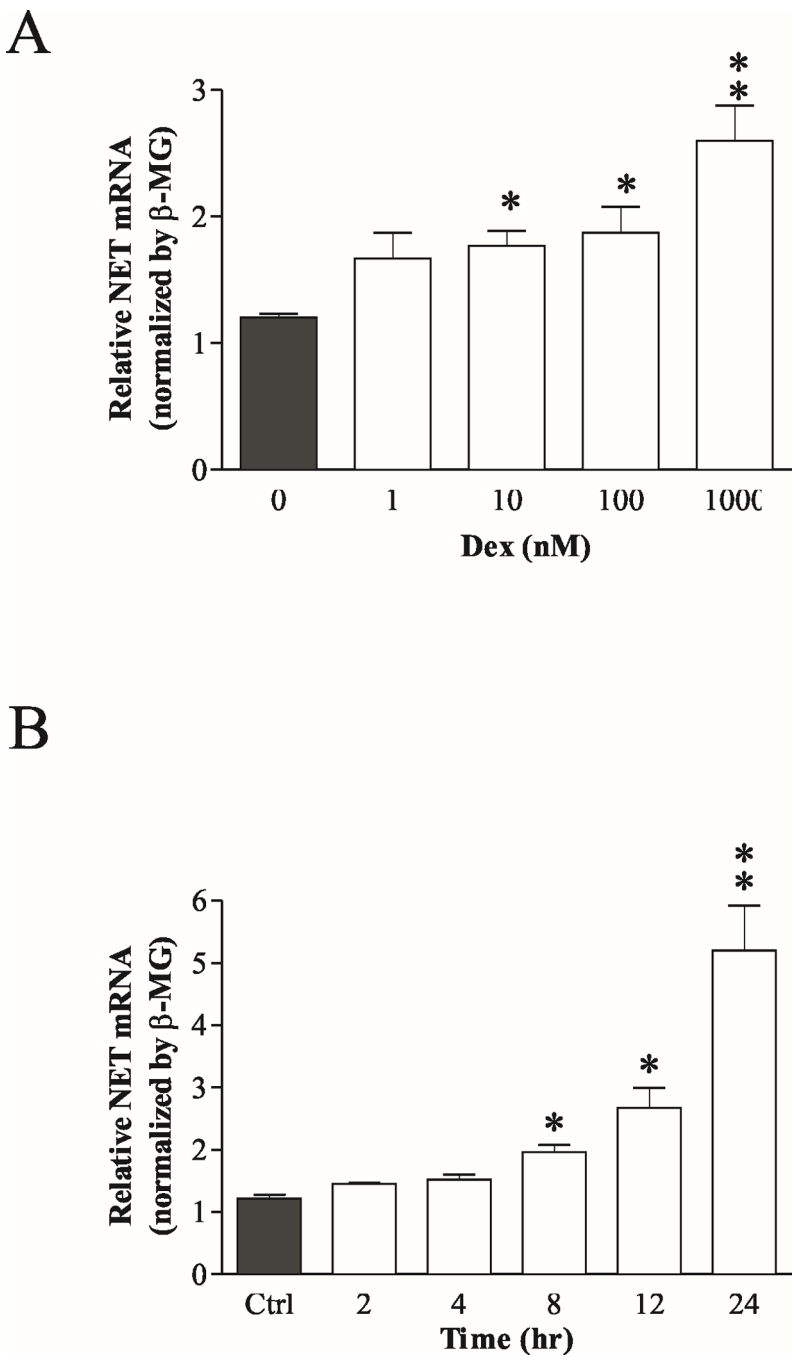


Figure 2.

Concentration- and time-dependence of NET mRNA levels after Dex exposure. SK-N-BE(2)M17 cells were treated with different concentrations of Dex (A) for 8 hr and 10 nM Dex for indicated times (B). The cells were harvested for real-time PCR with β-microglobulin (β-MG) as the internal control. For time course (B) the control was harvested at 4 hr time point. The data represent means ± SEM of three independent experiments. *$p < 0.05$, ** $p < 0.01$; compared to the group treated with vehicle (0) and control group (Ctrl).

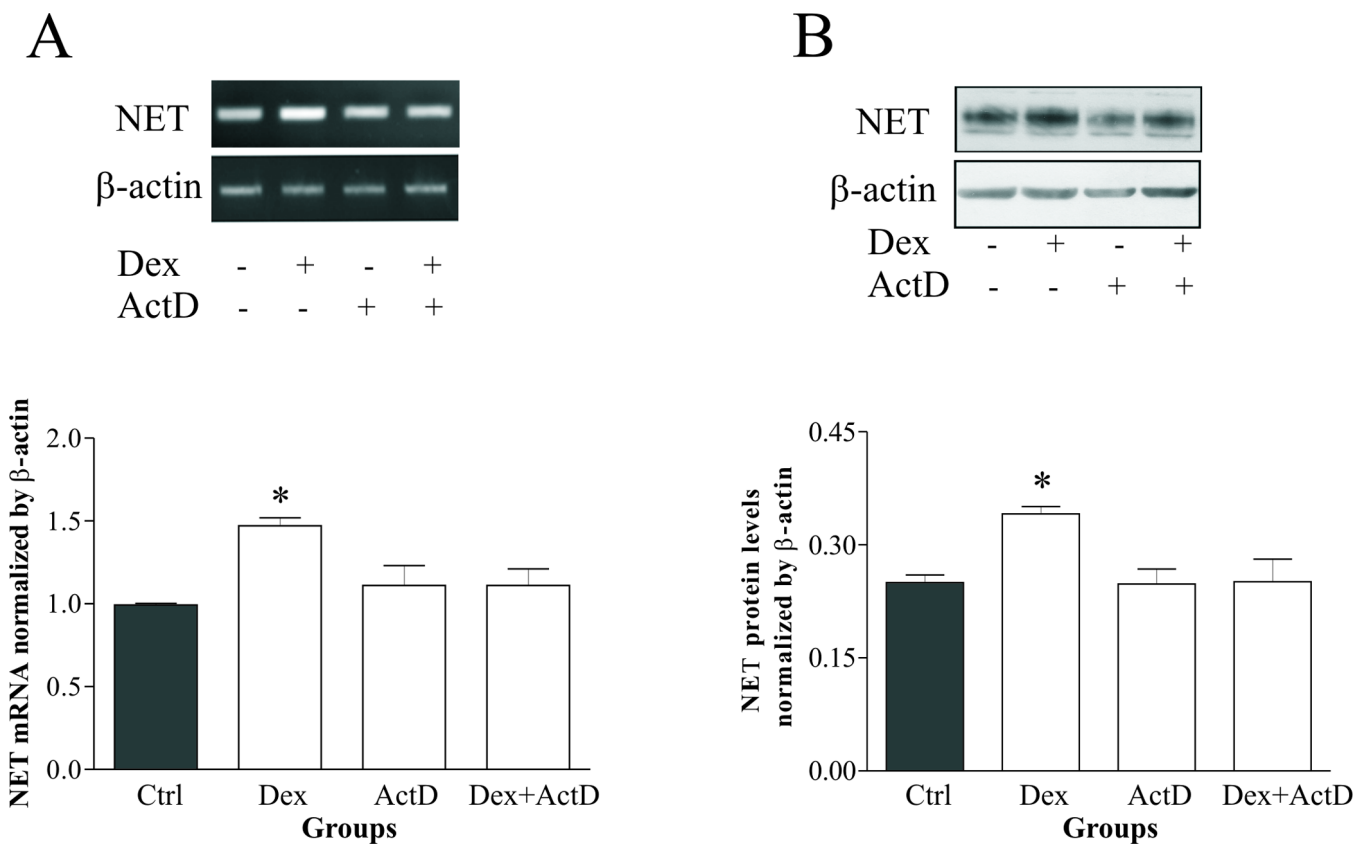


Figure 3.

Actinomycin treatment blocks Dex from inducing NET expression. SK-N-BE(2)M17 cells were pretreated with ActD (0.5 μM) for 30 min before treatment with 10 nM Dex for 8 hr. NET mRNA (A) and protein levels (B) were determined by RT-PCR and Western blotting, respectively. The upper panel shows autoradiograph obtained by RT-PCR or Western blotting of NET. The lower panel shows quantitative analysis of band densities. Values of NET bands were normalized to those of β-actin probed in each blot. The data represent means ± SEM of three independent experiments. *$p < 0.05$, compared to control groups. Ctrl: control; Dex: treated with dexamethasone; ActD: treated with actinomycin; Dex+ActD: treated with dexamethasone and actinomycin.

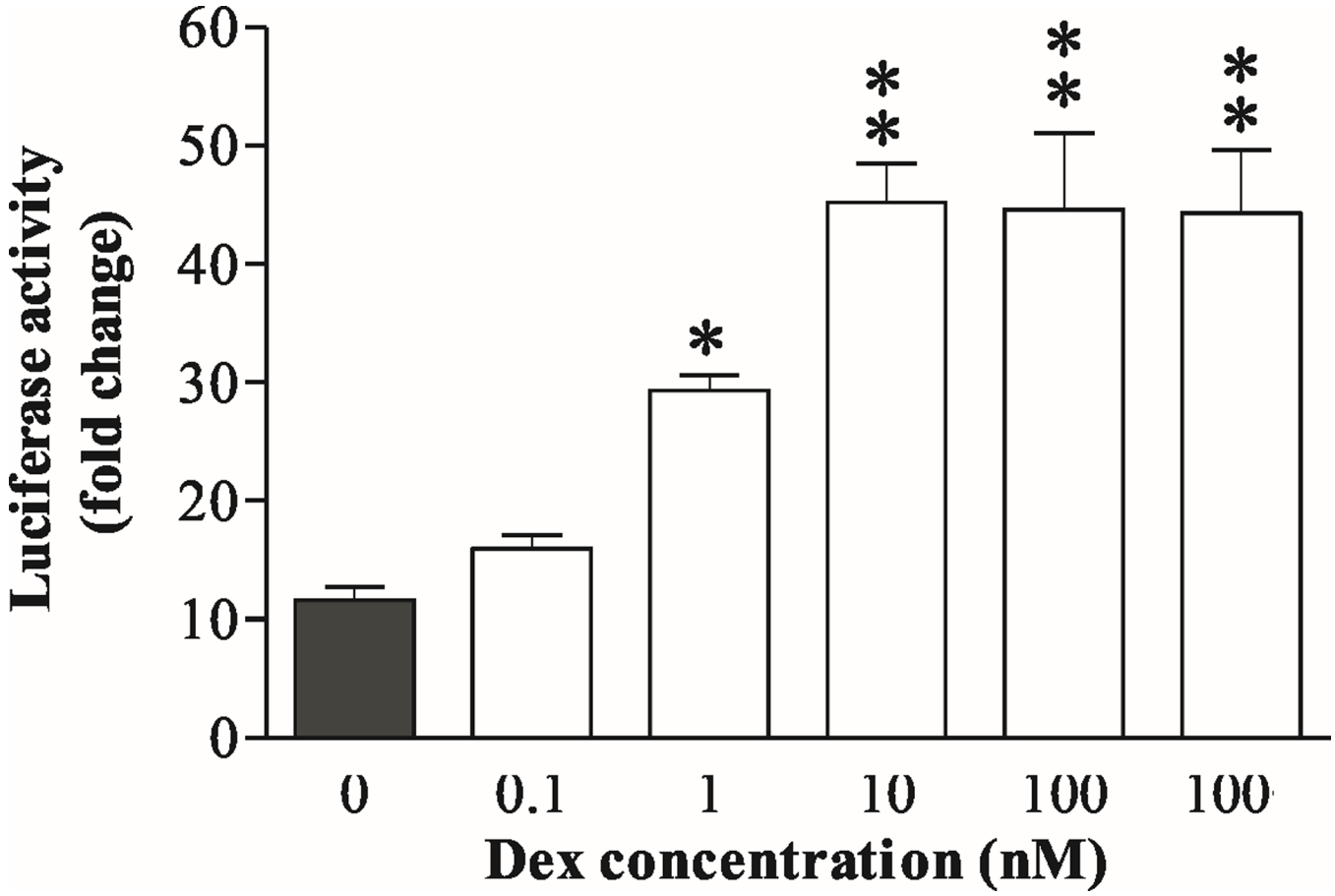


Figure 4.

Effect of Dex exposure on luciferase activity in SK-N-BE(2)M17 cells transfected with the reporter gene construct. Cells were transfected with pGL-hNET-F1 and maintained in serum-supplemented medium for 24 hr. They were then treated with vehicle or indicated concentrations of Dex for another 24 hr. Relative luciferease activity of each expression construct was measured as firefly luciferase/renila luciferase and expressed as fold change from pGL3-basic vector transfected cells (0). The data represent means ± SEM of three independent experiments. *$p < 0.05$, ** $p < 0.001$; compared to the group treated with vehicle (0).

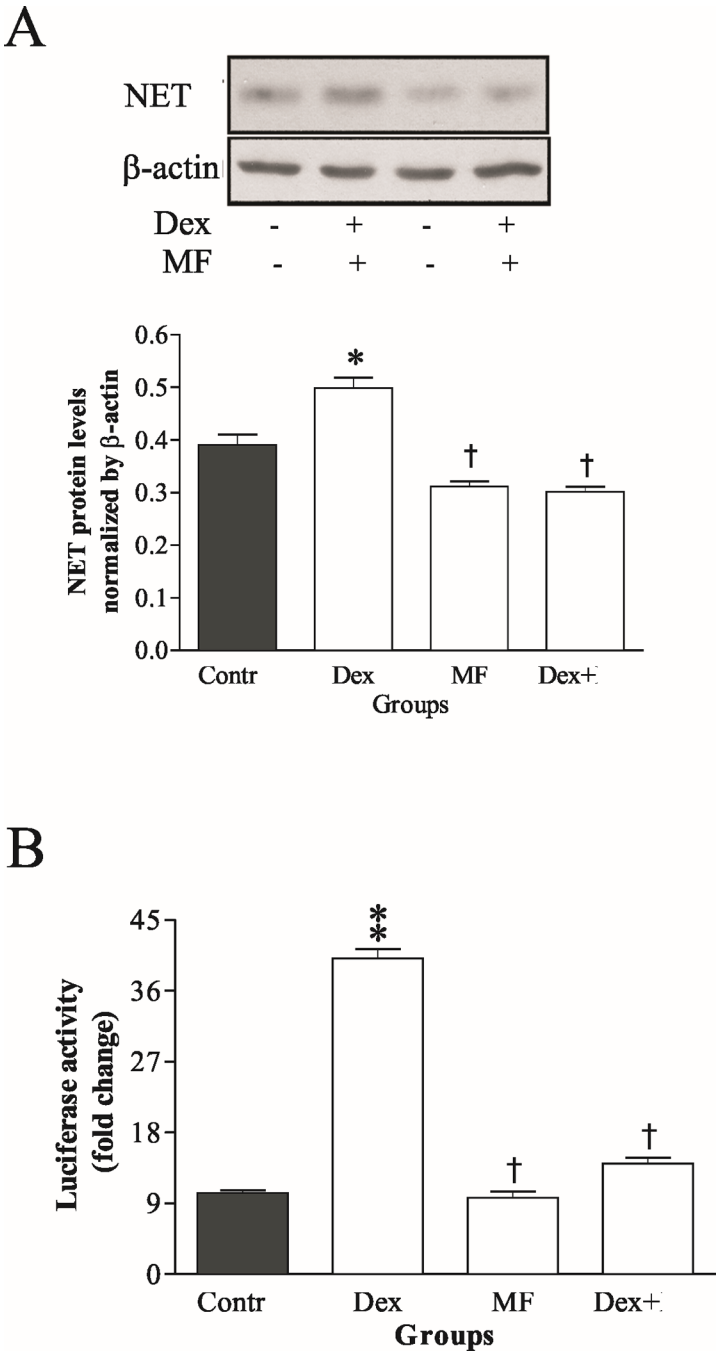
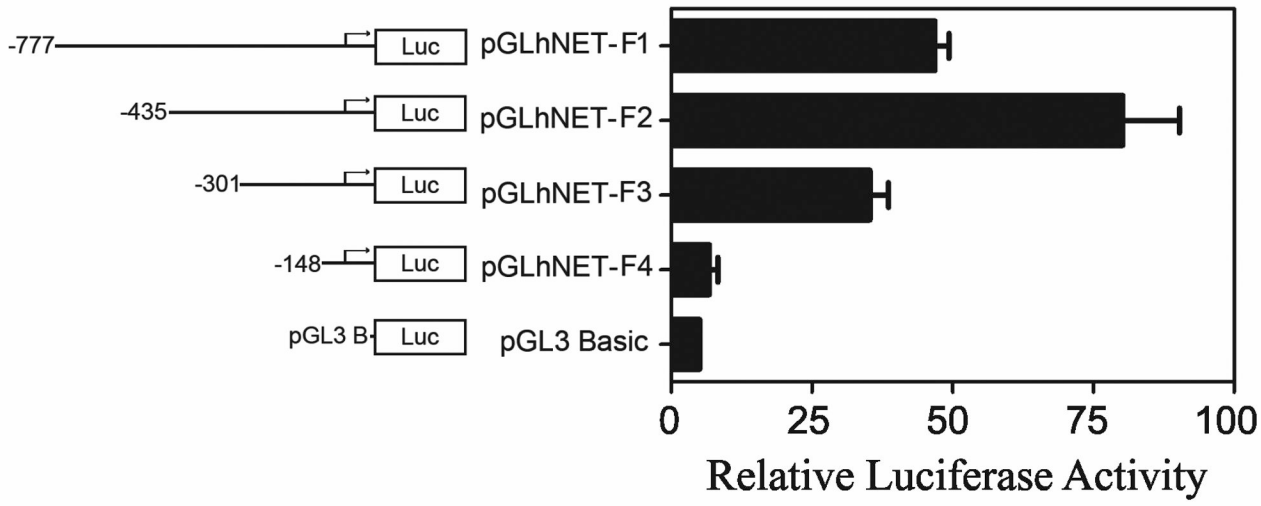


Figure 5.

Mifepristone (MF, 1 μM) prevented Dex (10 nM) from increasing NET protein levels (A) and activating the NET promoter (B). SK-N-BE(2)M17 cells and cells transfected with the reporter gene construct pGLhNET-F1 were treated with Dex and mifepristone, alone or in combination for 24 hr. NET protein levels were measured by Western blotting with the upper panel showing autoradiograph image and lower panel showing quantitative analysis of band densities. See Fig. 4 for luciferase activity measurement. Data represent the mean ± SEM of three independent experiments. *$p < 0.05$, ** $p < 0.01$; compared to control groups. † *$p<0.01$*, compared to the Dex group. Dex: treated with dexamethasone; MF: treated with mifepristone; Dex+MF: treated with dexamethasone and mifepristone.

A



B

The core C/EBP-β response element in human NET gene promoter

(-310) C/EBP-β

5'-AGAGTGGGTGAACGAGGAAAAG**TGCTGCAA**GGTCTTC
AGCCGCCCCCAGAGGGCTGTCAGAAGTCTCCAA-3'

(-240)

Figure 6.

A: Identification of Dex-responsive regions on the human NET gene promoter by serial deletion analysis. Cells were transfected with corresponding constructs and then treated with Dex for 24 hr. See Fig. 4 for luciferase activity measurement. Data are means ± SEM of three independent experiments. B: The core C/EBP-β response element, the *cis*-element of C/EBP-β, in the NET gene promoter.

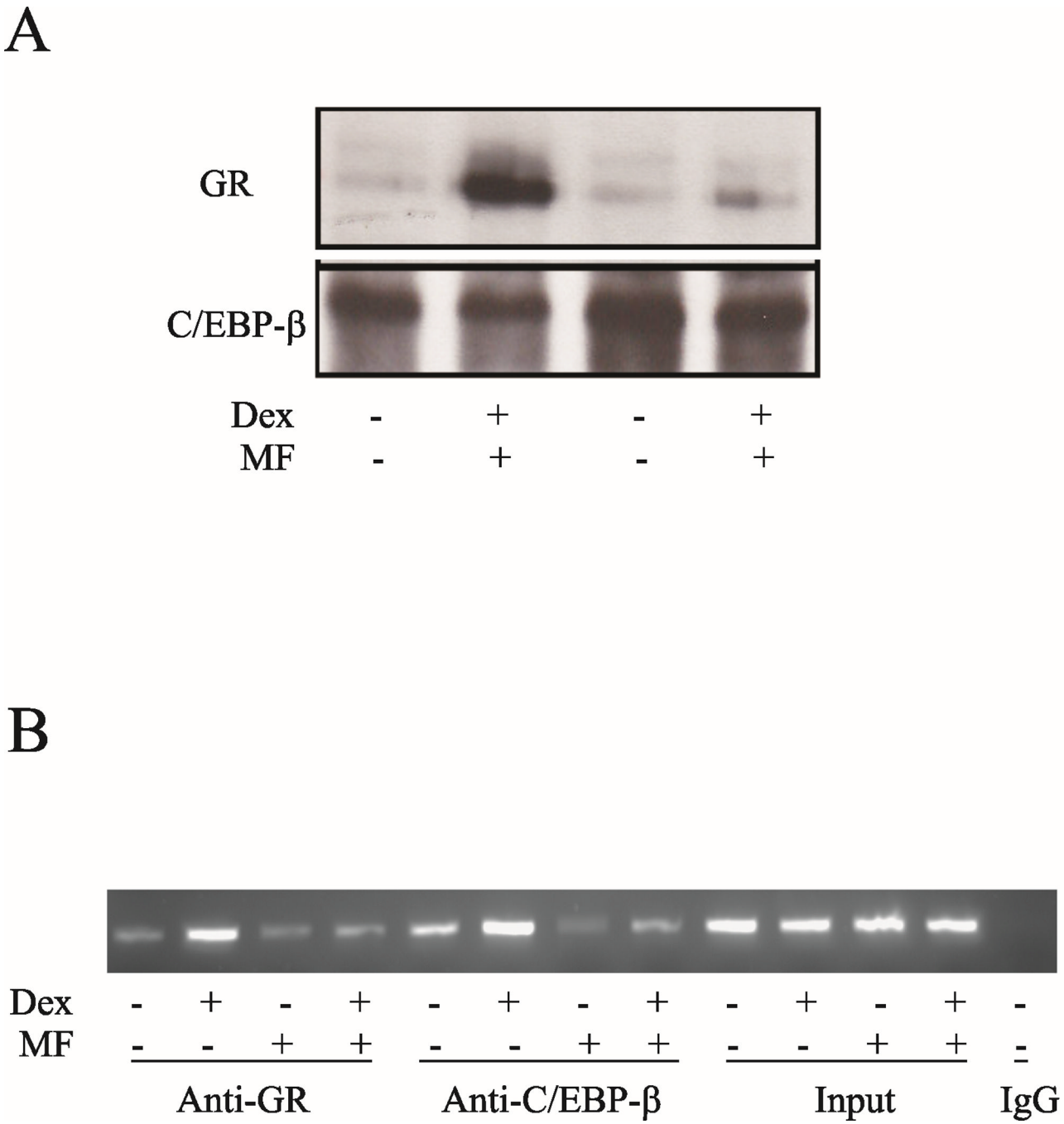


Figure 7.

A: The GR interacts with transcription factor C/EBP-β. SK-N-BE(2)M17 cells were treated with vehicle or 10 nM Dex in the absence or presence of 1 μM mifepristone (MF) for 24 hr. Nuclear proteins were extracted for immunoprecipitation using antibodies against C/EBP-β. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis for Western blotting with antibodies against GR (top) or C/EBP-β (bottom). Data are representative of three independent experiments. B: The GR and C/EBP-β bind to NET promoter. SK-N-BE(2)M17 cells were also treated with vehicle or 10 nM Dex in the absence or presence of 1 μM MF for 24 hr. The DNA extracted after immunoprecipitation of IgG, GR or C/EBP-β were used as templates for PCR (using the primers which were used to construct pGLhNET-

F3 fragment). “Input” serves as a loading control and rabbit IgG immunoprecipitation serves as a negative control. Band indicates the fragment of the NET promoter bound with GR or C/EBP-β in response to Dex treatment. Data are representative of three independent experiments.

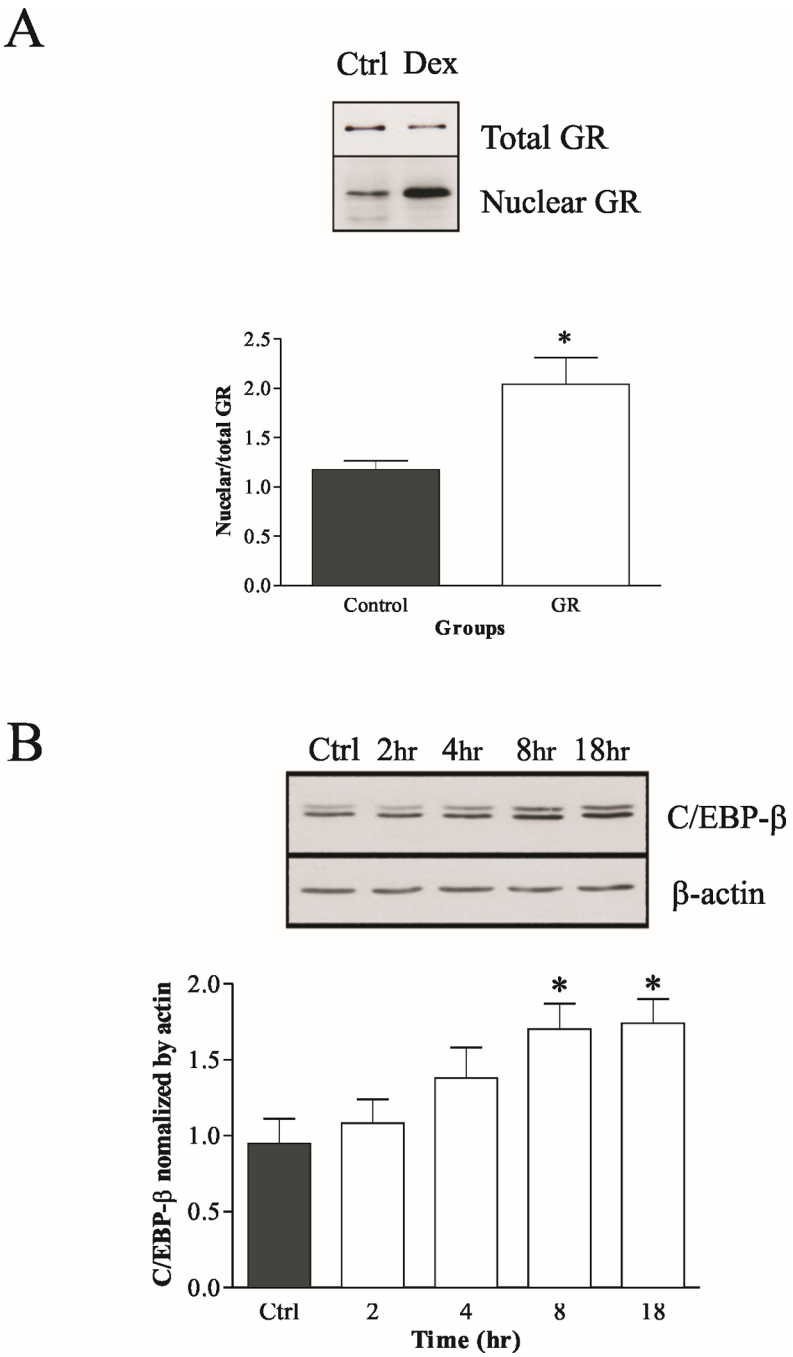


Figure 8.

The GR increases nuclear GR and C/EBP-β protein expression. A. SK-N-BE(2)M17 cells were treated with vehicle or 10 nM Dex for 8 h. Total proteins and nuclear proteins were harvested for measurement of GR proteins by Western blotting. B. SK-N-BE(2)M17 cell lysates were prepared following treatment with 10 nM Dex for the indicated times. Protein extracts were immunoblotted with antibody against C/EBP-β. Blots are respresentative of three experiments. The upper panel of each figure shows autoradiograph obtained by Western blotting for GR or C/EBP-β. The lower panel shows quantitative analysis of band densities for GR or C/EBP-β (45 kDa). *$p < 0.05$; compared to control groups (Ctrl).

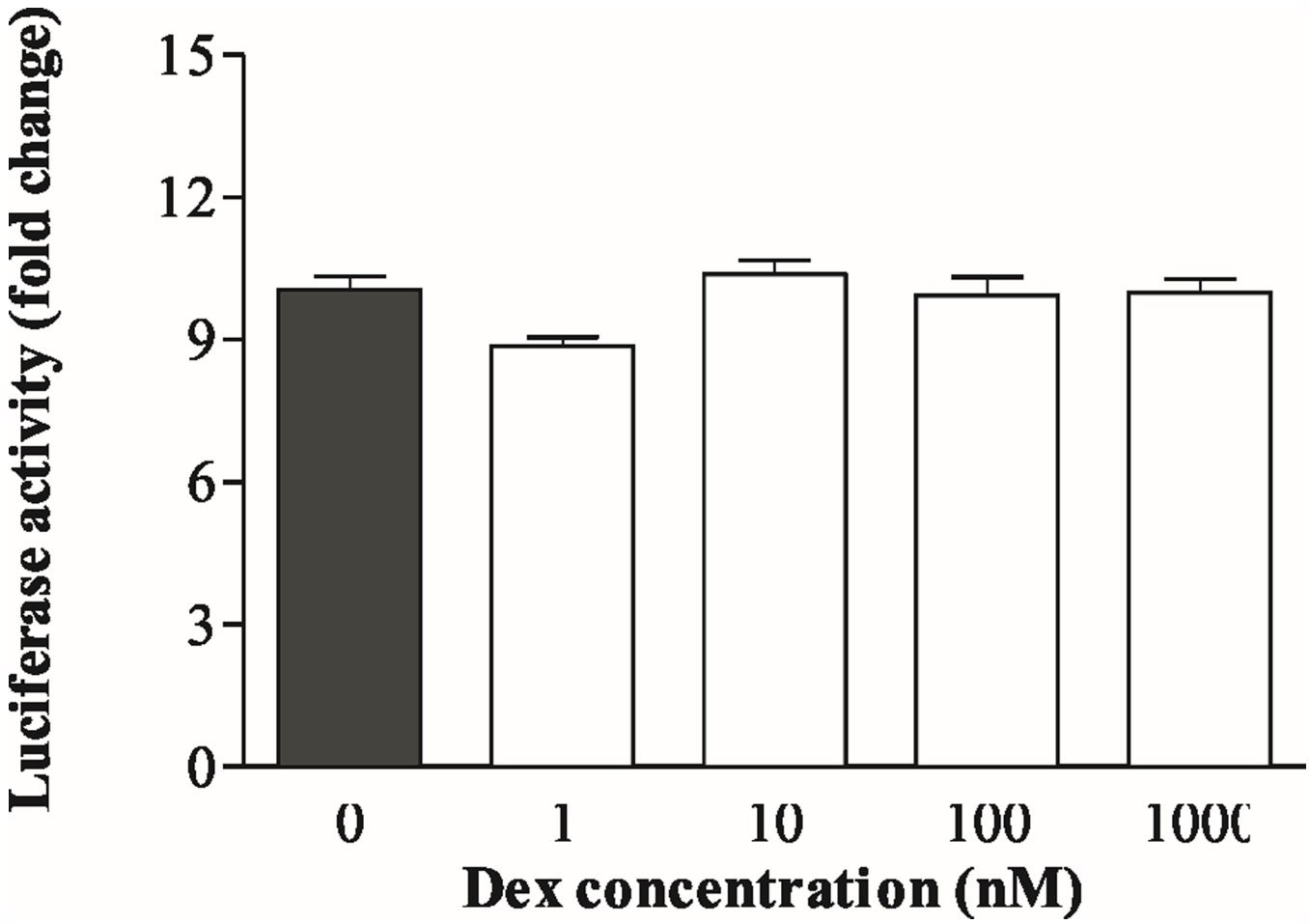


Figure 9.

The C/EBP-β response element is necessary for Dex-induced NET transactivation. Site-directed mutagenesis of the C/EBP-β response element site in the NET promoter abrogates Dex-induced luciferease responses. SK-N-BE(2) M17 cells were transiently transfected with mutated construct pGLhΔNET–F1 and treated for 24 hr with different concentrations of Dex. Cell extracts were assayed for luciferase activity, which was normalized to Renilla luciferase activity. Data represent the mean ± SEM of three independent experiments.