Role of proto-oncogenes in the regulation of proenkephalin mRNA expression induced by repeated nicotine injections in rat adrenal medulla

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Abstract

We have studied the effect of repeated systemic administrations of nicotine (3 mg/kg) at 30 min intervals on proenkephalin (proENK) mRNA level in rat adrenal gland. Northern blot analysis has shown that proENK mRNA expression was enhanced by repeated nicotine administrations. Additionally, repeated administrations of nicotine transiently induced the c-fos and c-jun mRNA levels after the first-third nicotine administration, and the c-fos and c-jun mRNA levels were returned to the basal level after the seventh administration of nicotine. c-Fos, c-Jun and Fra-2 protein levels were persistently increased until the seventh administration. The repeated nicotine administrations also elevated phospho-CREB without altering total CREB level in all tested groups. Immunohistochemical analysis showed that the increase of c-Fos and c-Jun proteins by repeated nicotine administrations is mostly medulla specific, while Fra-2 immuno reactivity was shown both in medulla and cortex. The repeated nicotine administrations enhanced the AP-1 and ENKCRE-2 DNA binding activities. Furthermore, the cross-competition studies revealed that the AP-1 proteins, rather than CREB, actively bind to ENKCRE-2 DNA domain. These results suggest that proENK
mRNA expression induced by repeated nicotine administrations may be mediated by AP-1 proteins, such as c-Fos, c-Jun and Fra-2 rather than CREB via interacting to the ENKCRE-2 DNA binding domain in rat adrenal medulla. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Adrenal medulla; Nicotine; Proenkephalin; Proto-oncogenes; Rat

Introduction

[Met\textsuperscript{5}]enkephalin (ME) in adrenal chromaffin cells is co-stored and co-released with catecholamines[1,2]. The secretion of ME or catecholamines can be regulated by activity of splanchnic nerve, which releases acetylcholine as the major neurotransmitter in the adrenal medulla. The existence of nicotinic receptors on adrenal medullary chromaffin cells has raised the possibility that the cholinergic neural input to the adrenal medulla regulates ME and catecholamine secretion. Indeed, the effects of nicotine on ME and catecholamine secretion have been investigated in vitro as well as in vivo [3–6]. In addition, the regulation of the proENK and tyrosine hydroxylase genes in the adrenal medulla has been studied under the stress or insulin-induced hypoglycemia [7,8] as well as in vitro using nicotine [6,9].

The c-Fos and Fos-related antigens (Fra) are inducible transcriptional factors that dimerize with the jun transcriptional factors to form AP-1 transcription complexes [10]. In adrenal medullary chromaffin cells, these transcriptional factor complexes recognize the AP-1 DNA element in the promoter regions of target genes, such as tyrosine hydroxylase, phenylethanolamine N-methyltransferase, and proENK, and modulate gene transcription through the expression of AP-1 proteins [9]. In addition, cyclic AMP (cAMP) response element binding protein (CREB) would also be expected to alter the expression of specific target gene in adrenal medulla, such as tyrosine hydroxylase [11], through interacting with CRE or CRE-like DNA element without altering its DNA binding activity through the phosphorylation by the protein kinases such as protein kinase A (PKA), calcium/calmodulin dependent protein kinase II, and IV [12,13]. Indeed, the proENK gene contains enkephalin cAMP response element like (ENKCRE-2) sequence in its promoter region, which may play a role in its own gene expression through interacting with proto-oncoproteins such as Fos and Jun as well as CREB [14–16]. However, the exact regulatory roles of AP-1 proteins and CREB underlying the proENK gene expression have not been well characterized in repeated nicotine administered rat adrenal medulla.

In an attempt to understand the involvement of proto-oncoproteins or CREB in proENK gene expression in vivo, the present study was designed to define the time-course of effect of repeated administration of nicotine on proto-oncoproteins, CREB, and proENK mRNA level using northern, western blot, and immunohistochemical analyses, and to examine the time course action of proto-oncoproteins on AP-1 and ENKCRE-2 DNA element using on electrophoretic mobility shift assay (EMSA).
Materials and methods

Treatment of animals

Male Sprague-Dawley rats (Dae-Han Animal Center, Dae-Ku, Korea) weighing 200-280 g were used. Animals were housed 2 per group in a room maintained at 22 ± 5 °C with an alternating 12 hr light-dark cycle. Handled rats were injected daily for 3-4 days to reduce stress during experimental treatment. Nicotine (Sigma, MO, USA) was dissolved in phosphate buffered saline (10 mM potassium phosphate, pH 7.5, 150 mM NaCl). Animals were administered with nicotine intraperitoneally at a dose of 3 mg per kg at 30 min interval and sacrificed 30 min after the first, third, fifth, seventh administration.

Isolation of total RNA and protein

Total cellular RNA was extracted from pooled rat adrenals (n=3/group) using a rapid guanidine thiocyanate-water saturated phenol/chloroform extraction and subsequent precipitation with acidic sodium acetate [17]. Total cellular RNA in the aqueous phase was precipitated with ice-cold isopropyl alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 nm and 280 nm. The separated organic layer was extracted twice with an equal volume of sterilized (Millipore) water and proteins were precipitated by adding 2 volumes of absolute ethanol to the water-extracted organic phase. The protein pellets were washed twice with cold absolute ethanol and dried. The dried pellets were dissolved in a denaturing buffer [6 M guanidium chloride, 20 mM Tris-HCl (pH 8.0), and 1 mM EDTA]. The protein samples were dialyzed against a renaturing buffer [20 mM Tris-HCl (pH 8.0) 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl2, 0.4 mM phenylmethylsulfonyl fluoride, and 20% glycerol] at 4 °C. The concentration of protein was determined with the Coomassie blue protein assay reagent (Pierce Chemical, Rockford, IL) using bovine serum albumin as the standard.

Non-isotopic Northern blot analysis

Total RNA (5 µg) was denatured and electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon Hybond-N hybridization membrane sheets (Amersham, Buckinghamshire, England). After baking for 1-2 hr at 80 °C, the membranes were prehybridized at 68 °C for at least 1 hr in prehybridization buffer (5×SSC, 50% formamide, 0.02% SDS, 0.1% sodium N-lauroyl sarcosine, and 2% blocking reagent). The digoxigenin (DIG)-labeled proENK, c-fos and c-jun probes were added to prehybridization buffer containing 50% formamide. The membranes were incubated overnight at 68 °C in a shaking water bath, and washed twice for 10 min per wash in 2×SSC and 0.1% SDS at room temperature. Then, the membranes were washed twice for 15 min per wash 0.1×SSC and 0.1% SDS. After equilibrating the membranes in Buffer I [100 mM maleic acid and 150 mM NaCl (pH 7.5)] for 1 min, the membranes were gently agitated in Buffer II (1% blocking reagent in Buffer I) for 30-60 min. The membranes were hybridized with the diluted (1:10,000)
anti-DIG-alkaline phosphatase (Boehringer Mannheim) in Buffer II for 30 min. After washing the membranes twice for 15 min per wash in 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in Buffer III [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM MgCl₂] for 2 min. Diluted CSPD (Boehringer Mannheim) (1:100 dilution in Buffer III) was spread over the surface of the membranes. After incubation of the membranes at 37 °C for 15-20 min, the membranes were exposed to Hyperfilm-ECL (Amer-
sham) for detection of the chemiluminescent signal. For rehybridization, blots were washed for 20 min at room temperature in sterilized (Millipore) water, then the membranes were washed for overnight at 65 °C in 50 mM Tris-HCl (pH 8.0), 50% dimethylformamide, and 1% SDS to remove the hybridized probe and rehybridized to the DIG-labeled rat cyclophilin cRNA probe, a gene encoding peptidyl-prolyl cis-trans isomerase which is constitutively expressed in most mammalian tissues with the exception of skeletal muscle [18,19]. The cRNA probes for proENK [20], c-fos [21], c-jun [22], and cyclophilin [18] were synthesized in vitro from linearized expression vectors with DIG-UTP, as suggested by the manufacturer (Boehringer Mannheim).

Western blot analysis

Total cellular protein (50 μg) was separated by an electrophoresis in 12% polyacrylamide gels. A pre-stained rainbow protein mixture (Amersham, Arlington Heights, IL) was used as the molecular weight standard. Electro-transferred to polyvinylidene difluoride filters were first blocked with blocking buffer [3% skim milk, 1% BSA, 10 mM Trizma base (pH 8.0), and 150 mM NaCl] and then incubated with antisera against c-Fos (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), Fra-2 (1:1000) (Santa Cruz Biotechnology), c-Jun (1:1000) (Santa Cruz Biotechnology), Actin (1:2000) (Santa Cruz Biotechnology), CREB (1:1000) (New England Biolabs, Beverly, MA), and phospho-CREB (1:1000) (New England Biolabs) in blocking buffer for 4 hr at room temperature. Filters were then washed 3 times with Tris-buffered saline containing 0.3% Tween-20 [TBST; 10 mM Trizma base (pH 8.0), 150 mM NaCl, and 0.3% Tween-20] for 5 min and then incubated with the anti-rabbit donkey IgG-horseradish peroxidase conjugate (1:5000) in blocking buffer at room temperature for 1 hr. After washing the filters with TBST for 10 min (3 times), ECL-plus solution (Amersham Life Science, England) were added. Then, the membranes were exposed to Hyperfilm-ECL (Amersham) for detection of light emission.

Immunohistochemistry

Three animals from each group were anesthetized with 50 mg/kg pentobarbital and then perfused transcardially with a 150 ml of 0.9% NaCl followed by 500 ml of 4% paraformal-dehyde in PBS. The adrenals were dissected and post-fixed overnight in the same solution at 4 °C. Then adrenals were immersed in 25% sucrose in PBS for 1 day and cut at 15 μm thickness with a cryostat. Adrenal sections were incubated first in H₂O₂ and in 2% normal goat serum for 60 min each and then immersed in primary antibodies, such as c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA), Fra-2 (Santa Cruz Biotechnology), c-Jun (Santa Cruz
Biotechnology), CREB (New England Biolabs, Beverly, MA), and phospho-CREB (New England Biolabs) at a dilution 1:500 for 48-72 hr. After a brief wash the sections were incubated in biotinylated anti-rabbit IgG for 2 hr (working dilution 1:1000; Boehringer Mannheim) and then, after brief wash, in streptavidin-peroxidase conjugate (dilution 1:4000; Boehringer Mannheim) for 2 hr at room temperature. The reaction complex on the section was visualized in a solution of 0.05% 3,3'-diaminobenzidine (Sigma) and 0.01% H₂O₂. Sections were mounted on gelatin-coated slides, air dried, dehydrated, and coverslipped.

Non-isotopic electrophoretic mobility-shift assay

The AP-1 (5'-CGC-ATG-CTACAGCCG-GAA-3'; Santa Cruz Biotechnology) and ENKCRE-2 (5'-CTA-GTG-ATG-CGTCAGCCG-GATC-3'; Korea Biotech, Dae-Ku, Korea) were annealed by incubating an equal molar concentration of each single-stranded oligonucleotide in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 200 mM NaCl at 95 °C for 10 min, and then the mixture was allowed to cool to room temperature. The DNA-binding assay followed the instructions in the manual provided with the DIG-Gel Shift Kit (Boehringer Mannheim). Binding reactions were carried out at room temperature for 20 min and reaction mixtures contained 50 μg of total protein, 20 mM HEPES (pH 7.6), 30 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.2% Tween20, 50 mg/ml poly (dI-dC), and

![Graph](image)

Fig. 1. The time course of proENK mRNA expression induced by repeated nicotine administrations. Northern blot analysis for proenkephalin (proENK) was performed using 5 μg of total mRNAs extracted from the whole adrenals of rats which were administered with nicotine intraperitoneally at a dose of 3 mg per kg at 30 min intervals and sacrificed 30 min after the first (N1), third (N3), fifth (N5), and seventh (N7) administration. The constitutively expressed mRNA encoding cyclophilin (CPN) was used as an internal loading control. The vertical bars in upper panel indicate the S.E. (*p<0.05; **p<0.01 compared to the control group; n=3 independent experiments).
approximately 0.3 pmol of specified probe labeled with DIG-ddUTP using terminal deoxynucleotidyl transferase (Boehringer Mannheim). Protein-DNA complexes were separated from protein-free DNA by non-denaturing electrophoresis in 5% polyacrylamide (30:1, acrylamide:bisacrylamide) gels. Gels were run at room temperature in 89 mM Trizma base (pH 8.0), 89 mM boric acid, and 2 mM EDTA at a constant voltage (8 V/cm), and electroblotted onto positively charged nylon membranes. The membranes were baked at 80°C for 15 min, washed with 0.3% Tween 20 in Buffer I, and hybridized with the diluted (1:10,000) anti-DIG-alkaline phosphatase in Buffer II for 30 min. After two washes for 15 min with 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in Buffer III for 2 min. The method for detection of chemiluminescence was identical to the method used for the non-isotopic Northern blot analysis.

Statistical Analysis

The expressions of c-fos, c-jun, and proENK mRNA induced by nicotine was quantified with Bio-profile Bio-1D application (Vilber-Lourmat, France), and expressed as the percentage of the control mRNA levels. One-way analysis of variance (ANOVA) was used to test for overall statistical significance. Multiple comparisons between groups were made using Fisher’s Least Significance Test.

Results

Effect of Nicotine on proENK mRNA level

All data shown in the present study were repeated at least three-times. The proENK mRNA and protein levels in rat adrenal gland were examined 30 min after the first, third, fifth, and seventh administration of nicotine (3 mg per kg). As shown in Fig. 1, the proENK mRNA level began to increase significantly after the third administration (about 1.8-fold as much as control group) and further increased after the seventh administration (about 2.3-fold as much as control group).

Effect of nicotine on c-fos and c-jun mRNA levels

Northern blot assay showed that c-fos mRNA level was elevated transiently after the first administration of nicotine (about 2-fold as much as control group) and returned to the basal level.
level after the seventh administration of nicotine (Fig. 2A). The significant induction of c-jun mRNA level was detected after the first administration of nicotine, peaked after the fifth administration (about 3-fold as much as control group), and began to decrease after the seventh administration (Fig. 2B).
Effect of nicotine on proto-oncoproteins levels

Western blot analyses of proto-oncoproteins were carried out using protein extracted from the whole adrenal gland to determine whether the levels of c-Fos, Fra-2 and c-Jun were correlated with the expression of proENK mRNA induced by repeated nicotine adminis-

![Figure 3](image-url)

Fig. 3. The time course of c-Fos, Fra-2, and c-Jun protein expression induced by repeated nicotine administrations. (A) Western blot analysis for c-Fos, Fra-2, and c-Jun was performed using 50 μg of total proteins extracted from the whole adrenals of rats which were administered with nicotine intraperitoneally at a dose of 3 mg per kg at 30 min intervals and sacrificed 30 min after the first (N1), third (N3), fifth (N5), and seventh (N7) administration. (B) Adrenal sections (medulla and cortex) were immuno-stained using antisera against c-Fos, Fra-2, and c-Jun in control, N1, and N7 rats. Polyclonal antisera against each proteins were used at a 1:1000 dilution for Western blot and 1:500 dilution for immunostaining.
trations. The repeated administrations of nicotine transiently increased c-Fos protein. The c-Fos, c-Jun and Fra-2 protein levels began to increase after the first administration of nicotine, and further increased through the seventh administration of nicotine. (Fig. 3A). Immunohistochemical analysis showed that the increases of c-Fos and c-Jun proteins induced by repeated nicotine administrations were mostly occurred in the medulla rather than the cortex (Fig. 3B). In contrast to c-Fos and c-Jun proteins, repeated nicotine administrations induced the Fra-2

Fig. 4. The time course of CREB and phospho-CREB protein expression induced by repeated nicotine administrations. (A) Western blot analysis for cAMP response element binding protein (CREB) and phosphorylated (Ser133) CREB (phospho-CREB) was performed using 50 μg of total proteins extracted from the whole rat adrenals which were administered with nicotine intraperitoneally at a dose of 3 mg per kg at 30 min intervals and sacrificed 30 min after the first (N1), third (N3), fifth (N5), and seventh (N7) administration. (B) Adrenal sections (medulla and cortex) were immuno-stained using antisera against CREB and phospho-CREB in control, N1, and N7 rats. Polyclonal antisera against each proteins were used at a 1:2000 dilution for Western blot and 1:500 dilution for immunostaining.
protein in both medulla and cortex. The immunostaining against these proteins was found only in the nuclei.

**Effect of nicotine on CREB and phospho-CREB levels**

Western blot analyses for CREB and phospho-CREB (phosphorylated at Ser\(^{133}\)) levels were also performed. Repeated nicotine administrations did not affect the basal level of total CREB in any tested group (Fig. 4A). However, the phospho-CREB level was markedly increased after 1 hr, and this increase was maintained until the seventh administration. In immunohistochemical analysis, highly quantity of CREB was found in the medulla rather than the cortex, and repeated nicotine administrations did not affect CREB level (Fig. 4B). However, repeated nicotine administrations markedly increased phospho-CREB level mostly in the medulla rather than the cortex.

![Fig. 5. The time course of ENKCRE-2 and AP-1 DNA-binding activities induced by repeated nicotine administrations. Electrophoretic mobility-shift gel assays as described under Experimental Procedures was performed using total proteins extracted from the whole adrenals of rats which were administered with nicotine intraperitoneally at a dose of 3 mg per kg at 30 min intervals and sacrificed 30 min after the first (N1), third (N3), fifth (N5), and seventh (N7) administration. Fifty \(\mu\)g of total protein were used for determining AP-1 and ENKCRE-2 DNA-binding activities. For self- or cross-competition studies, the electrophoretic mobility-shift assays were performed in the presence or absence of a 50-fold molar excess of the unlabeled double-stranded oligonucleotide (AP-1, CRE, or ENKCRE-2) using proteins from N7 group.](image-url)
**Effect of nicotine on AP-1 or ENKCRE-2 DNA binding activity**

An electrophoretic mobility shift assay using AP-1 and ENKCRE-2 DNA oligomers was carried out to examine whether there were correlations between the expressions of proENK mRNA and proto-oncoprotein, and the phosphorylation of CREB protein. Both AP-1 and ENKCRE-2 DNA binding activities began to increase after the first administration and had a plateau after the third administration of nicotine (Fig. 5). The diminutions of AP-1 and ENKCRE-2 DNA binding activities by self-competition with 50× excess of unlabeled (cold) probes indicate that these binding activities were specific. The cross-competitions between AP-1 and ENKCRE-2 showed that the AP-1 or ENKCRE-2 DNA binding activity was also diminished by 50× unlabeled cross-competitor.

**Discussion**

This study has dealt with the activation of proENK mRNA expression induced by repeated nicotine administration in relation to proto-oncogene expression, such as c-Fos, Fra-2, and c-Jun, and phosphorylation of CREB in the rat adrenal gland. Repeated treatments with nicotine at 30 min interval enhanced the expression of proENK mRNA level in rat adrenals. This increase was maintained and enhanced until the seventh administration. In a preliminary experiment before this study, proENK mRNA expression was not change by saline-treated group (7 times injection/30 min interval) compare with intact group in rat adrenal gland (data not shown). Thus, the increased expression of proENK mRNA could be due to nicotine action but not stress after repeated injection. Although whole adrenal was used in extraction of total mRNA in the present study, the increase of proENK transcript was presumed to be originating from the medulla because previous studies have demonstrated that the expression of proENK is localized in epinephrine-producing adrenal chromaffin cells rather than cortex [23]. This finding is consistent with several previous studies that repeated nicotine administrations (3 mg/kg×7 injections over 3.5 h) increase ME in rat adrenals [24].

To characterize the regulatory factors, which are responsible for the increased proENK mRNA expression induced by repeated nicotine administration, we first examined the effect of nicotine on the expression of AP-1 proteins, such as Fos and Jun. Indeed, among the cis-regulatory elements, such as ENKCRE-1, ENKCRE-2, and AP-2, in the promoter region of proENK [14,25–27], the ENKCRE-2 element could confer responsiveness to both phorbol esters and activators of the cAMP dependent pathways [14], suggesting that ENKCRE-2 acts as an AP-1 and CRE element. In Northern blot analysis, repeated nicotine administrations transiently increased c-fos and c-jun mRNA levels with different profile in the time course study. The level of c-fos mRNA is maximally increased by nicotine after the first administration while c-jun mRNA was continuously increased, suggesting that c-fos gene expression is more rapidly responded to nicotine stimulation.

The expression of c-Fos, c-Jun and Fra-2 protein levels induced by repeated nicotine administrations was persistently enhanced through the seventh administration. Furthermore, repeated nicotine administrations did not alter the basal expression of Fra-1 protein level (data...
not shown). The increases of c-Fos and c-Jun proteins induced by repeated nicotine administrations were mostly occurred in the medulla rather than the cortex. In contrast, Fra-2 protein was detected in both medulla and cortex. Weidenfeld et al. (1989) [28] reported that stimulation of nicotinic receptor in the brain activate adrenal cortex. In this point, systemically administered nicotine may stimulate nicotinic receptor in the brain and subsequently activated Fra-2 expression in the adrenal cortex. Although further study is required to elucidate the mechanism involved in nicotine-induced increase Fra-2 protein in the adrenocortical region, the results of the present study suggest that nicotine may change a certain gene expressions in rat adrenocortical region as well as medulla through the activation of transcriptional factor, such as Fra-2.

Our results showed that c-Fos, c-Jun and Fra-2 protein expressions in adrenal medulla are well correlated with the increase of proENK mRNA level induced by repeated nicotine administrations. This finding in vivo study is in line with previous in vitro study that c-Fos, c-Jun, or Fra-2 protein actively participates in the regulation of proENK mRNA expression in cultured bovine adrenal chromaffin cells [29]. In addition to AP-1 proteins, CREB would be also expected to alter the expression of proENK gene via an ENKCRE-2 DNA element in its promoter region. Indeed, Konradi et al. (1993) [16] had reported that the haloperidol-induced increase of proENK mRNA level in the striatal region of the rat brain is mainly regulated by CREB rather than c-Fos. CREB is known to alter the expression of specific target genes containing CRE and CRE-like DNA elements through a phosphorylation process [12]. Therefore, we examined the level of phosphorylated CREB protein. In Western blot analysis, we found that repeated nicotine administrations also increase phospho-CREB after the first administration, which is maintained through the seventh administration, without altering total CREB level. In the immunohistochemical analysis, the increase of phospho-CREB level induced by repeated nicotine administrations mostly occurred in the medulla rather than the cortex. Although this result suggests that repeated nicotine administration may selectively activate CREB protein in the medulla rather than the cortex, this response may be also due to the abundance of total CREB protein in medulla than in cortex. Although the underlying physiological role of highly expressed adrenal medullary CREB is currently unknown, a large quantity of CREB protein level in adrenal medulla may be helpful to dynamically change certain CREB-dependent gene expressions, such as c-fos and tyrosine hydroxylase [30,31].

To elucidate the involvement of AP-1 proteins, such as c-Fos, c-Jun, and Fra-2 in the regulation of proENK mRNA expression induced by repeated nicotine administrations, an electrophoretic mobility shift assay against AP-1 or ENKCRE-2 DNA sequence was examined. AP-1 DNA binding activity began to be elevated after the first administration of nicotine and the elevation of AP-1 DNA binding activity was maintained through the seventh administration. Thus, the effect of repeated nicotine administration on AP-1 DNA binding activity was well correlated with the proENK mRNA expression. Previous studies have demonstrated that a single or acute repeated (3 mg/kg i.p., 7 injections equi-spaced over a 3 h period) nicotine administrations decreased AP-1 DNA binding activity [24,32]. Although the discrepancy in the AP-1 DNA binding responses to repeated nicotine administrations is not currently understood, it may be due to the difference in the genetic background of animals tested. In addition to AP-1 DNA binding activity, ENKCRE-2 DNA binding activity was also
induced by repeated nicotine administrations and was well correlated with AP-1. Furthermore, cross competition assay showed that AP-1 and ENKCRE-2 DNA binding activity were diminished by unlabeled cross competitors, suggesting that AP-1 DNA binding proteins, such as c-Fos, c-Jun, and Fra-2, actively participate in the regulation of ENKCRE-2 DNA binding activity induced by repeated nicotine administrations.

Taken together, the results of the present study strongly suggest that proENK mRNA expression was enhanced by AP-1 proteins such as c-Fos, c-Jun, and Fra-2 via interacting to ENKCRE-2 DNA binding domain in the repeated nicotine administered rat adrenal medulla. In addition, although CREB itself appears not to be participated in the increase of ENKCRE-2 DNA binding activity induced by repeated nicotine administrations, there are some evidence to support the possible involvement of CREB on the regulation of proENK mRNA expression through the formation of heterodimer with c-Jun protein, of which levels were elevated by repeated nicotine administrations. Indeed, c-Jun protein has been demonstrated to have a leucine zipper that can bind to CREB [33].

Although the present study focused on the role of AP-1 and CREB protein in the regulation of proENK gene expression in vivo, there were several lines of evidence that certain factors such as AP-2, AP-4, NFκB, and NF-1 also bind to the proENK promoter region and, in turn, regulate the expression of the proENK gene [34,35]. In addition, the proENK gene promoter has a series of homologous transcriptional control domains, such as ENKCRE-1, ENKCRE-2, β1, β2, and AP-2 in humans [14,36] and rats [25–27]. Therefore, the molecular mechanisms involved in proENK gene expression may not be as simple as recognition of enhancer domains by AP-1. The exact nature of the interactions between these transcription factors and the proENK gene expression in the repeated nicotine administered rat adrenal medulla needs to be determined.

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