Cycloheximide Increases Proenkephalin and Tyrosine Hydroxylase Gene Expression in Rat Adrenal Medulla

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ABSTRACT
The effect of cycloheximide (CHX; 5 mg/kg) on proenkephalin (proENK) and tyrosine hydroxylase (TH) mRNA expression in rat central and peripheral nervous systems was studied. CHX increased proENK and TH mRNA levels in the adrenal gland, but not in hippocampus, striatum, midbrain, brainstem, pituitary, and hypothalamus. The pretreatment with actinomycin D (0.5 mg/kg) significantly decreased CHX-induced proENK and TH mRNA expression, suggesting that the CHX-dependent increase of these mRNA levels may be caused by the increase of transcriptional activity rather than RNA stabilization. To investigate the factors involved in CHX-induced proENK and TH mRNA expression, the effect of CHX on activator protein-1 (AP-1), cAMP response element (CRE) binding protein (CREB), and glucocorticoid response element (GRE) was tested. In AP-1, the basal expression of Fra-2 and c-Jun proteins and AP-1 DNA binding activity in the adrenal medulla was higher than other tissues tested, but CHX reduced these protein levels and AP-1 DNA binding activity. In CREB, CHX time dependently increased the level of phospho-CREB without altering total CRE level and CRE DNA binding activity. Furthermore, phospho-CREB actively participated in CRE DNA binding activity. In GRE, although CHX increased plasma and adrenal corticosterone level, RU486 (10 mg/kg) reduced CHX-induced proENK, but not TH, mRNA level in a partial manner. These results suggest that the basal expression of proENK and TH mRNA transcription in the adrenal gland seems to be tonically inhibited by de novo protein synthesis. In addition, CHX-dependent increase of proENK and TH mRNA expression in the adrenal medulla is well correlated with phospho-CREB level, but not TH, mRNA expression in the adrenal medulla.

[Met⁵]enkephalin (ME) in the adrenal chromaffin cells is costored and coreleased with catecholamines (Viveros et al., 1979). The secretion of ME or catecholamines has been reported to be regulated by activity of splanchnic nerve, which releases acetylcholine as a major neurotransmitter in the adrenal medulla. The secretion of ME and catecholamines is tightly regulated through the regulation of gene expression of their precursor form, proenkephalin (proENK), and rate-limiting enzyme, tyrosine hydroxylase (TH). The expression of these genes in the adrenal medulla has been reported to be regulated by direct neural (acetylcholine) and hormonal (glucocorticoid) inputs to the adrenal medullary cells (Stachowiak et al., 1990).

There are common features in the regulation of proENK and TH that are caused by the similarity of cis-elements, such as activator protein-1 (AP-1) and the cAMP response element (CRE), in their promoter/enhancer region. In rodent adrenal medulla, AP-1 and CRE play important roles in the regulation of proENK and TH gene expression that are mediated by interaction with AP-1 transcriptional factors (Fos/Jun) and CRE binding protein (CREB) (Konradi et al., 1993; Bacher et al., 1996; Won et al., 1997). AP-1 proteins are functionally active when Fos:Jun or Jun:Jun dimers are formed (Morgan and Curran, 1991). In addition, CREB has been reported to alter the expression of proENK and TH in the adrenal medulla activity (Konradi et al., 1993; Tinti et al., 1996) through phosphorylation without altering its DNA binding activity (Gonzalez and Montminy, 1989; Sheng et al., 1991). In a proENK gene promoter region, ENKCRE-2 has been reported to play a role in its own gene expression through the interaction with CREB as well as AP-1 proteins because of its sequence similarity to AP-1 and CRE (Comb et al., 1986; Sonnenberg et al., 1989; Konradi et al., 1993). Several in vitro and in vivo studies have demonstrated that

ABBREVIATIONS: ME, [Met⁵]enkephalin; proENK, proenkephalin; TH, tyrosine hydroxylase; AP-1, activator protein-1; CRE, cAMP response element; CREB, CRE-binding protein; GRE, glucocorticoid response element; CHX, cycloheximide; p38, p38 mitogen-activated kinase; JNK, c-Jun NH₂-terminal kinase, ERK, extracellular signal response kinase; SSC, standard saline citrate; DIG, digoxigenin; TBS, Tris-buffered saline; AMD, actinomycin D; ATF-2, activating transcription factor-2; MAPK mitogen-activated protein kinase.
Experimental Procedures

**Treatment of Animals.** Male Sprague-Dawley rats (Dae-Han Animal Center, Dae-Ku, Korea) weighing 200 to 280 g were used. Animals were housed two per group in a room maintained at 22 ± 5°C with an alternating 12-h light/dark cycle. Rats were handled daily for 3 to 4 days to reduce stress during experimental treatment. CHX (5 mg/kg; Sigma Chemical, St. Louis, MO) was dissolved in PBS (10 mM potassium phosphate, pH 7.5, 150 mM NaCl). RU486 (3 mg/kg; BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) was dissolved in corn oil. Both drugs were administered i.p. to animals.

**Isolation of Total RNA and Nonisotopic Northern Blot Analysis.** Total cellular RNA was extracted using a rapid guanidine thiocyanate-water saturated phenol/chloroform extraction and subsequent precipitation with acidic sodium acetate (Chomczynski and Sacchi, 1987). Total cellular RNA in the aqueous phase was precipitated with ice-cold isopropyl alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 and 280 nm. 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 to 2 h at 80°C, the membranes were prehybridized at 68°C for at least 1 h in prehybridization buffer (5× standard saline citrate (SSC), 50% formamide, 0.02% SDS, 0.1% sodium N-lauroyl sarcosine, and 2% blocking reagent). The digoxigenin (DIG)-labeled proENK and TH 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 in 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 to 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 disodium 3-(4-methoxyphenoxy) (1,2-dioxetane-3,2′-5′-chloro)tricyclo[3.3.1.13,7]decan-4-yl) phenolphosphat (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 to 20 min, the membranes were exposed to Hyperfilm-enhanced chemiluminescence (Amersham) 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 overnight at 65°C in 50 mM Tris-HCl, pH 8.0, 50% dimethylformamide, and 1% SDS to remove the hybridized probe and rehybridize to the DIG-labeled rat cyclophilin cRNA probe, a gene encoding peptidyl-prolyl cis-trans-isomerase, which is constitutively expressed in most mammalian tissues, except for skeletal muscle (Danielson et al., 1988). The cRNA probes for proENK (Yoshikawa et al., 1984), TH (Grima et al., 1985), and cyclophilin (Danielson et al., 1988) were synthesized in vitro from linearized expression vectors with DIG-UTP, as suggested by the manufactuer (Boehringer Mannheim).

**Isolation of Total Proteins and Western Blot Analysis.** To reduce the dephosphorylation of phosphokinases, the pooled rat adrenal gland (n = 3/group) was rapidly broken down by sonication in 1× SDS loading buffer and boiled. After centrifugation (15,000g, 10 min), the concentration of protein was determined with the detergent compatible protein assay kit (Bio-Rad, Richmond, CA). Total cellular proteins (50 μg) were separated by electrophoresis in 12% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride filters. After blocking with 3% skim milk in Tris-buffered saline (TBS; 10 mM trizma base, pH 8.0, 150 mM NaCl), the filter was then allowed to interact with antisera against c-Fos, Fra-1, Fra-2, FosB, c-Jun, JunB, and JunD (all from Santa Cruz Biochemicals, Santa Cruz, CA), and CREB, phospho-CREB, phospho-ATF-2, phospho-ERK1/2, phospho-p38, and phospho-JNK (all from New England Biolabs, Beverly, MA) in a blocking buffer for 4 h at room temperature. Filters were washed three times with TBS containing 0.3% Tween-20 for 5 min and then reacted with anti-rabbit donkey IgG conjugated with horseradish peroxidase for 1 h. After washing three times with TBS containing 0.3% Tween-20 (3×), the filters were then allowed to interact with antisera against c-Fos, Fra-1, Fra-2, FosB, c-Jun, JunB, and JunD (all from Santa Cruz Biochemicals, Santa Cruz, CA), and CREB, phospho-CREB, phospho-ATF-2, phospho-ERK1/2, phospho-p38, and phospho-JNK (all from New England Biolabs, Beverly, MA) in a blocking buffer for 4 h at room temperature. Filters were washed three times with TBS containing 0.3% Tween-20 (3×), the filters were then allowed to interact with enhanced chemiluminescence-plus solution and exposed to X-ray film.

**Immunohistochemistry of the Adrenal Gland.** Animals were anesthetized with 50 mg/kg pentobarbital and then perfused transcardially with 150 ml of 0.9% NaCl followed by 500 ml of 4% paraformaldehyde in PBS. The adrenal glands were dissected and postfixied overnight in the same solution at 4°C. Then the adrenal
glands were immersed in 25% sucrose in PBS for 1 day and cut at 15 μm thickness with a cryostat. The adrenal gland sections were incubated first in H2O2 and in 2% normal goat serum for 60 min each and then immersed in primary antibodies, such as Fra-2, c-Jun, and JunD at a 1:500 dilution for 48 h. After a brief wash, the sections were incubated in biotinylated anti-rabbit IgG for 2 h (working dilution 1:1000; Boehringer Mannheim) and, after brief wash, in streptavidin-peroxidase conjugate (dilution 1:4000; Boehringer Mannheim) for 2 h 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 cover-slipped.

**Preparation of Nuclear Extracts and Nonisotopic Electrophoretic Mobility-Shift Assay.** Nuclear extract was prepared using the published method with slight modification (Dignam et al., 1983). Tissues were minced, washed twice with ice-cold TBS, lysed in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl2, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin) containing 0.1% Nonidet P-40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 5,000g for 5 min. The pellet nuclei were washed with buffer A without Nonidet P-40 and resuspended in 40 μl of buffer B [20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.12 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin]. After 30 min on ice, lysates were centrifuged at 15,000g for 15 min. Supernatant containing the nuclear proteins was diluted with modified buffer C [20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 0.05 M KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol] and stored at −70°C until used. Nuclear proteins were used for the electrophoretic mobility shift assay for detection of AP-1 and CRE DNA binding activity. The double strands of AP-1 (5′-CTG-AGC-CGG-CGG-GAA-3′; Santa Cruz Biotechnology) and CRE (5′-ATG-ACT-CAG-CCG-GAA-3′; Santa Cruz Biotechnology) were annealed by incubating an equal molar concentration of each single-stranded oligonucleotide in 10 mM Tris pH 8.0, 1 mM EDTA, and 200 mM NaCl at 95°C for 10 min; then the mixture was allowed to cool to room temperature. DNA-protein binding reactions were carried out at room temperature for 20 min, and reaction mixtures contained 10 μg of total protein, 10 mM Tris base, pH 7.9, 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 1 μg poly(dI-dC), 5% (v/v) glycerol, and approximately 0.3 pmol of specified probe labeled with DIG-dUTP using terminal deoxynucleotidyl transferase (Boehringer Mannheim). Protein-DNA complexes were separated from protein-free DNA by nondenaturing electrophoresis in 5% polyacrylamide (30:1, acrylamide/bisacrylamide) gels. Gels were run at room temperature in 50 mM Tris, pH 8.3, 0.38 M glycine, and 2 mM EDTA, and electrobotted onto positively charged nylon membranes. The membranes were UV-crosslinked, washed with 0.3% Tween 20 in buffer I, and hybridized with the diluted anti-DIG-alkaline phosphatase [1:10,000 (75 mM/ml)] in Buffer II for 30 min. After washing five times for 15 min with 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. The method for detection of chemiluminescence was identical with the method used for the nonisotopic Northern blot analysis. For supershift assay, 2 μg of antibodies against CREB and phosphorylated CREB were added into the DNA-protein binding reaction mixture 1 h before adding DIG-labeled probes.

**Determination of Corticosterone.** Plasma and adrenal gland corticosterone levels were determined fluorometrically according to a method published previously (Levine et al., 1967). Briefly, the blood sample from retro-orbital venous plexus was collected into a heparin-treated microcentrifuge tube and centrifuged. The adrenal medulla sample was blended and centrifuged. Supernatant (50 μl) was added to 5 ml of methylene chloride and incubated at room temperature for 10 min. After filtration with cheese cloth, the mixture was combined with 2.5 ml of fluorescence reagent (7:3, sulfuric acid/absolute ethanol), vortexed vigorously, and incubated for 30 min at room temperature. After centrifugation, the lower layer was fluorometrically measured (excited wavelength, 475 nm; emission wavelength, 530 nm).

**Results**

The **Time- and Dose-Dependent Effect of CHX on ProENK mRNA Expression in Rat Central and Peripheral Nervous System.** All data shown in this study were from experiments repeated at least three times. To examine the effect of CHX on central and peripheral nervous systems, Northern blot analysis of proENK mRNA was performed at various doses (0.5, 1, 2, and 5 mg/kg at a time of 6 h) and time points (0.5, 1, 3, and 6 h at a dose of 5 mg/kg) in the hypothalamus, pituitary, striatum, hippocampus, midbrain, brainstem, and adrenal gland. As shown in Fig. 1A, the proENK mRNA level was not affected in the hypothalamus, striatum, hippocampus, midbrain, and brainstem. In the pituitary gland, the basal expression of proENK mRNA was dose and time dependently reduced by treatment with CHX. In the adrenal gland, however, the basal expression of proENK mRNA level was increased dose and time dependently by treatment with CHX; the induction of proENK mRNA expression began to increase significantly at a dose of 1 mg/kg and 0.5 h after CHX treatment (Fig. 1B). At a dose of 5 mg/kg, proENK mRNA level was increased about 20-fold as much as the control group at 6 h after CHX treatment. To rule out the possibility that the increase of proENK mRNA level seen with CHX was caused by its stabilization, actinomycin D (AMD; 0.5 mg/kg), an RNA synthesis inhibitor, was administered 30 min before CHX. As shown in Fig. 1C, the pretreatment of AMD significantly attenuated the increase of proENK mRNA level induced by CHX (about 1.9-fold decrease), although AMD alone did not affect the basal level of proENK mRNA.

The **Time- and Dose-Dependent Effect of CHX on TH mRNA Expression in the Rat Adrenal Gland.** Northern blot analysis of TH mRNA was performed in the adrenal gland. As shown in Fig. 2, the expression of TH mRNA level was dose and time dependently increased by the treatment with CHX; the induction of TH mRNA expression began to increase significantly at a dose of 1 mg/kg and 1 h after CHX treatment. At a dose of 5 mg/kg, TH mRNA level was increased 4-fold as much as the control group at 6 h after CHX treatment. To rule out the possibility that the increase of TH mRNA level seen with CHX was due to its stabilization, AMD was preadministered 30 min before CHX. As shown in Fig. 2B, the pretreatment of AMD significantly attenuated the increase of TH mRNA level induced by CHX (about 1.6-fold decrease), although AMD alone did not affect the basal level of TH mRNA.

The **Basal Expression of AP-1 (Fos/Jun) in Rat Central and Peripheral Nervous System.** To investigate the possible role of pre-existing AP-1 in proENK and TH mRNA expression in CHX-treated animals, the basal levels of each AP-1 component and their binding activities to AP-1 DNA binding motif were examined. As shown in Fig. 3A, among the central and peripheral nervous tissues (hypothalamus, pituitary, striatum, hippocampus, midbrain, brainstem, and adrenal gland), the adrenal gland had a higher basal AP-1 DNA binding activity, whereas the activity in other tissues
was undetectable. To determine the composition of the basal AP-1 DNA binding activity in the adrenal gland, each AP-1 component (c-Fos, Fra-1, Fra-2, FosB, c-Jun, JunB, or JunD) level was examined (Fig. 3B). Western blot analysis showed that the basal c-Fos and JunB levels were undetectable in all tissues tested. The basal Fra-1 level was most abundant in the hippocampus. In the other tissues, such as hypothalamus, striatum, midbrain, and brainstem, smaller quantities of Fra-1 were also detected. Although the relative quantity was different according to tissues, the basal JunD level was most abundant in the adrenal glands, hippocampus, striatum, and brain stem. However, smaller quantities of JunD were also detected in other tissues such as hypothalamus, pituitary, and midbrain. In the hippocampus, striatum, midbrain, and brainstem, the basal level of FosB protein was also detected. In addition, the relative quantity of FosB-L (45-kDa long form FosB, a completely encoded FosB) and FosB-S (35-kDa shorter form FosB, a truncated form of FosB) (Nakabeppu and Nathans, 1991) was different according to tissues tested. In contrast to Fra-1, FosB, and JunD, the basal levels of Fra-2 and c-Jun were detected only in the adrenal gland. All of the highly expressed basal AP-1 proteins, such as Fra-2, c-Jun, and JunD, in the adrenal gland were medulla specific (Fig. 3C).

The Time-Dependent Effect of CHX on AP-1 and Its DNA Binding Activity in the Rat Adrenal Gland. To investigate the possible role of AP-1 in the regulation of CHX-induced proENK and TH mRNA expression, the time course effect of CHX on AP-1 DNA binding activity and each AP-1 component was examined. The basal AP-1 DNA binding activity was reduced by CHX treatment in a time-dependent manner (Fig. 4A). In addition, highly expressed basal Fra-2, c-Jun, and JunD levels were reduced by CHX. However, CHX did not affect the basal levels of c-Fos, Fra-1, FosB, and JunB proteins (Fig. 4B).

The Time-Dependent Effect of CHX on the Phosphorylation of Mitogen-Activated Protein Kinases, CREB, and Activating Transcription Factor-2 (ATF-2) in the Rat Adrenal Gland. To investigate CHX as a signaling agonist, the effect of CHX on the active phosphorylated form of three mitogen-activated protein kinases (MAPKs), such as ERK1/2, p38, and JNK1/2, and transcriptional factors, such as CREB, ATF-2, and c-Jun, was examined. As shown in Fig. 5A, CHX time dependently reduced the basal levels of phos-

![Fig. 1. The time- and dose-dependent effect of CHX on proENK mRNA expression in rat central and peripheral nervous system. A, Northern blot analysis was performed at the indicated doses (0.5, 1, 2, and 5 mg/kg, i.p., at a time of 6 h) and time points (0.5, 1, 3, and 6 h at a dose of 5 mg/kg, i.p.) in various tissues in central and peripheral nervous system, such as hypothalamus (HT), pituitary (PT), striatum (ST), hippocampus (HP), midbrain (MB), and brainstem (BS). B, Adrenal gland (AD). C, Northern blot analysis was performed at 6 h after i.p. administrations with saline-saline, saline-CHX (5 mg/kg), AMD-saline (0.5 mg/kg), and AMD-CHX. AMD was given for 30 min before the administration of CHX. Total RNA (5 μg) was used for determining proENK mRNA levels. The constitutively expressed mRNA encoding cyclophilin (CPN) was used as an internal loading control. The vertical bars in the column graph indicate the S.E.M. *P < .05; **P < .01; ***P < .001 compared with the control group; n = three independent experiments.](image-url)
pho-ERK1/2 and JNK1/2. However, the phospho-p38 level was markedly increased 0.5 h after CHX treatment. Even with the changes of these phosphorylated MAPK levels, the total amounts of ERK1/2, p38, and JNK were not affected up to 6 h after CHX treatment. The phospho-CREB level was also increased by CHX in a time-dependent manner without altering the total CREB level, but highly phosphorylated ATF-2 and the undetectable range of phospho-c-Jun levels were not affected by CHX at all time points tested (Fig. 5B). Similar to the level of total CREB, CRE DNA binding activity was not affected by CHX at all time points (Fig. 5C). In a supershift assay using the nuclear extract from the rats treated with CHX for 6 h, the antisera against phospho-CREB as well as total CREB caused a supershift of CRE DNA binding activity.

**The Time-Dependent Effect of CHX on Corticosterone Level in the Rat Adrenal Gland and Blood and the Effect of RU486 on CHX-Induced ProENK and TH mRNA Levels.** To assess the possible involvement of endogenous corticosterone in the regulation of CHX-induced proENK and TH mRNA expression, the effects of CHX on the adrenal gland corticosterone level and RU486 on CHX-induced proENK and TH mRNA expression were examined. As shown in Fig. 6A, CHX time dependently increased adrenal gland corticosterone level. The corticosterone level was significantly increased at 0.5 h and increased further up to 6 h after CHX treatment (about 8-fold as much as control level).

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**Fig. 2.** The time- and dose-dependent effect of CHX on TH mRNA expression in rat central and peripheral nervous system. A, Northern blot analysis was performed at the indicated doses (0.5, 1, 2, and 5 mg/kg) at a time of 6 h (data not shown) and time points (0.5, 1, 3, and 6 h at a dose of 5 mg/kg) in the adrenal gland (AD). B, the effects of AMD on CHX-induced TH mRNA Northern blot analysis was performed at 6 h after i.p. administrations with saline-saline, saline-CHX (5 mg/kg), AMD-saline (0.5 mg/kg), and AMD-CHX. AMD was given for 30 min before the administration of CHX. Total RNA (5 μg) was extracted for determining TH mRNA level. The constitutively expressed mRNA encoding cyclophilin (CPN) was used as an internal loading control. The vertical bars in the column graph indicate the S.E.M. *P < .05; **P < .01; ***P < .001 compared with the control group; n = three independent experiments.

**Fig. 3.** The basal expression of AP-1 proteins in central and peripheral nervous system. Gel shift (A) and Western blot analyses (B) were performed in various tissues, such as hypothalamus (HT), pituitary (PT), striatum (ST), hippocampus (HP), midbrain (MB), brainstem (BS), and adrenal gland (AD) from normal rats. For gel shift assay of AP-1 DNA binding activity, 5 μg of nuclear extract was used. Cellular protein (50 μg) was used for Western blot analysis of AP-1 proteins using antisera against c-Fos, Fra-1, Fra-2, FosB, c-Jun, JunD, and JunB (1:1000 dilution). C, Immunohistochemical analysis was performed in normal adrenal glands using antisera (Fra-2, c-Jun, and JunD; 1:500).

**Fig. 4.** The time-dependent effect of CHX on its DNA binding activity in rat adrenal gland. Gel shift (A) and Western blot analyses (B) were performed at the indicated time points (0.5, 1, 3, and 6 h at a dose of 5 mg/kg) in adrenal gland. For gel shift assay of AP-1 DNA binding activity, 5 μg of nuclear extract was used. Cellular protein (50 μg) was used for determining the AP-1 levels using antiserum against c-Fos, Fra-1, Fra-2, FosB, c-Jun, JunD, or JunB (1:1000 dilution).
The pretreatment with a steroid receptor antagonist, RU486, slightly but significantly reduced CHX-induced proENK mRNA expression (Fig. 6B). However, CHX-induced TH mRNA expression was not affected by the pretreatment with RU486.

**Discussion**

In this study, we have demonstrated the effect of CHX on proENK and TH mRNA expression in various central and peripheral tissues in vivo. Our previous study demonstrated that the pretreatment with CHX effectively reduced proENK and prodynorphin mRNA expressions induced by kainic acid in the rat hippocampus (Won et al., 1997). Similar to the observation in the hippocampus, the basal level of proENK mRNA in the pituitary gland was reduced by CHX treatment in a dose- and time-dependent manner. However, in the adrenal gland, CHX itself increased the proENK and TH mRNA level in a dose- or time-dependent manner. Additionally, no change in proENK mRNA expression in hippocampus, hypothalamus, striatum, midbrain, or brainstem was observed. To rule out the possibility that the increase of proENK and TH mRNA levels induced by CHX may be caused by their stabilization, AMD, an RNA synthesis inhibitor, was administered before CHX treatment. As shown in Figs. 1C and 2B, the pretreatment of AMD effectively attenuated the increases of proENK and TH mRNA levels induced by CHX, suggesting that CHX may increase proENK and TH mRNA levels via increasing transcriptional activities rather than mRNA stabilities. CHX-dependent increase of proENK and TH mRNA expressions have not ever been reported in other experiments both in vivo and in vitro, although there were reports that CHX further enhanced proENK mRNA levels in the presence of positive stimuli, such as prostaglandin E2 (Won et al., 1998b) and 8-bromo-cAMP (Theodoridou et al., 1994). In primary cultured astrocytes, CHX treatment further enhances the prostaglandin E2- or 8-bromo-cAMP-dependent induction of proENK mRNA expression. However, CHX seems not to be a direct enhancer according to the unchanged basal proENK mRNA expression in the presence of CHX alone. In the primary cultured bovine adrenal chromaffin cells, CHX alone did not affect the basal level of proENK (Mar et al., 1992; Suh et al., 1992), suggesting that, in contrast to in vitro systems, there are uncharacterized mechanisms in in vivo systems, such as CHX-induced changes in neural input to the adrenal, in the regulation of proENK and TH mRNA expression. However, the exact rea-

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**Fig. 5.** The time-dependent effect of CHX on the phosphorylation of MAPKs, CREB, and ATF-2 in rat adrenal gland. Western blot analyses of phosphorylated or total MAPKs (A), transcriptional factors (B), and gel shift analysis of CRE DNA binding activity (C) were performed at the indicated time points (0.5, 1, 3, and 6 h at a dose of 5 mg/kg) in the rat adrenal gland. Total cellular protein (50 μg) was used for determining total CREB level and phosphorylated ERK (p-ERK), total ERK, phosphorylated p38 (p-p38), total p38, phosphorylated JNK (p-JNK), total JNK, phosphorylated CREB (p-CREB), phosphorylated ATF-2 (p-ATF2), and phosphorylated c-Jun (p-c-Jun). Polyclonal antisera against these proteins were used at a 1:1000 dilution. For gel shift assay of CRE DNA binding activity, 5 μg of nuclear extract was used. For supershift assay, 2 μg of antiserum against CREB or phospho-CREB was added in DNA-protein binding mixture.
son for the CHX-dependent increase of proENK and TH mRNA level in the in vivo system has not been elucidated. To delineate the regulatory mechanism of CHX-dependent proENK and TH mRNA expression in the adrenal gland, we first examined the effect of CHX on AP-1. The promoter/enhancer region of proENK gene ENKCRE-2 has been reported to play an important role in the regulation of proENK transcription and can be recognized by AP-1 or CREB because ENKCRE-2 shares similarities with both the AP-1 and CRE recognition sequences (Comb et al., 1988; Kobierski et al., 1991). The promoter region of the TH gene also has an AP-1 DNA binding motif (Stachowiak et al., 1994) that has been reported to be involved in TH mRNA expression induced by certain stimuli, such as histamine (Faucon Biguet et al., 1991). As shown in Fig. 3, the AP-1 DNA binding activity in the adrenal gland was higher than that in other tissues tested in the normal rats. This AP-1 DNA binding activity in the adrenal gland seems to be composed of highly expressed c-Jun, Fra-2, and JunD proteins. Furthermore, these proteins were detected primarily in the medulla rather than the cortex layer. Because the protein synthesis inhibitor has been known as a potent activator of some transcriptional factors, such as c-Jun and CREB, through phosphorylation, it is speculated that CHX can phosphorylate the basally expressed AP-1 components if the half-lives of the basally expressed adrenal medulla Fra-2, c-Jun, and JunD are long enough for the activation of proENK and TH transcription. However, as shown in Fig. 4A and B, the basal AP-1 DNA binding activity and the basal protein levels of Fra-2, c-Jun, and JunD were time dependently reduced by CHX treatment. Furthermore, the phospho-c-Jun protein level was not affected by CHX at all time points tested. Therefore, this finding suggests that although Fra-2, c-Jun, and JunD proteins are highly expressed in the adrenal medulla, AP-1 proteins appear not to be involved in CHX-induced increases of proENK and TH mRNA expression.

A protein synthesis inhibitor has been known as a potent intracellular signaling agonist (Mahadevan and Edwards, 1991). To understand the signaling effect of CHX on the MAPK pathways, the effect of CHX on active phosphorylated forms of ERK1/2, p38, and JNK1/2 was tested in the adrenal gland. According to several previous studies, the activation of MAPKs may modulate the activities of several transcriptional factors that can affect the proENK and TH mRNA expression. ERK is known to phosphorylate p90 ribosomal S6 kinase, which is coupled with the phosphorylation of CREB (Bohm et al., 1995); p38 is known to phosphorylate ATF-2, which can interact with AP-1 and CRE DNA elements (Gupta et al., 1995; Livingstone et al., 1995); and JNK is known to phosphorylate c-Jun (Derijard et al., 1994). As shown in Fig. 5A, although CHX caused the time-dependent decrease of phospho-ERK1/2 and JNK1/2 levels, the phospho-p38 level was markedly increased by CHX at 0.5 h. These results were maintained up to 6 h after CHX treatment without altering the total enzyme levels. In contrast to phospho-p38, the basal phospho-ATF2 level was not affected by CHX at all time points tested. In addition, the treatment with CHX did not affect the phospho-c-Jun protein level. However, even the reduction of phospho-ERK1/2 and phospho-CREB levels was time dependently increased by CHX. Several signaling kinases, such as protein kinase A and calcium/calmodulin dependent protein kinases II and IV (Gonzalez and Montminy, 1989; Sheng et al., 1991), have been reported to be involved in the CREB phosphorylation. Therefore, rather than the ERKs, other signaling kinases seem to be involved in CHX-dependent CREB phosphorylation.

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![Fig. 6](image-url). The time-dependent effect of CHX on corticosterone level in the rat adrenal gland and blood, and the effect of RU486 on CHX-induced proENK and TH mRNA expressions. A, the level of corticosterone was quantified at the indicated time points (0.025, 0.5, 1, 3, and 6 h; at a dose of 5 mg/kg) in the rat adrenal gland and blood using a fluorometer. B, Northern blot analysis was performed at 6 h (at a dose of 5 mg/kg) in the adrenal gland administered with corn oil-saline, RU486 (RU; 10 mg/kg in corn oil)-saline, corn oil-CHX (5 mg/kg), and RU-CHX. RU was preadministered for 20 min before the administration with CHX. Total RNA (5 μg) was used for determining proENK and TH mRNA levels. The constitutively expressed mRNA encoding cyclophilin (CPN) was used as an internal loading control. The vertical bars in the column graph indicate the S.E.M. *P < .05; **P < .01; ***P < .001 compared with the control group; †P < .05 compared with the corn oil-CHX treated group; n = three independent experiments).
In the previous studies, CREB was reported to be a potent positive transcription factor for the regulation of proENK and TH mRNA expression. In the rat TH gene, CRE is essential for the basal and cAMP-stimulated transcription (Kim et al., 1993). In addition, the rat gene for proENK bears CRE-like sequences that play a role in striatal proENK mRNA up-regulation in vivo and in vitro (Konradi et al., 1993). As shown in Fig. 5B, in contrast to Fra-2, c-Jun, and JunD levels, CHX did not affect the total CREB protein level at any time points tested, which was well correlated with CRE DNA binding activity. Because CREB is known to alter the expression of specific target genes containing CRE and CRE-like DNA elements through the phosphorylation process rather than increasing its DNA binding activity (Gonzalez and Montminy, 1989; Arias et al., 1994), the increased phospho-CREB level may be enough to alter CRE-dependent gene expression without altering CRE DNA binding activity. Indeed, the supershift assay showed that CHX-induced phospho-CREB actively participated in the formation of CRE DNA binding activity. Therefore, our results suggest that CHX-dependent increase of the phospho-CREB level, rather than AP-1, seems to be involved in CHX-induced proENK and TH mRNA expression in the adrenal medulla. However, further study should be performed to determine whether CHX-induced phospho-CREB is enough for the activation of proENK and TH mRNA expression.

The adrenal cortex is a major source of several steroid hormones. Each layer (zona glomerulosa, zona fasciculata, or zona reticularis) of the adrenal cortex synthesizes and releases mineralocorticoids, glucocorticoids, or sex steroids, respectively. Among these steroid hormones, glucocorticoid has been reported to up-regulate the adrenal medullary TH (Fossum et al., 1992) and proENK (Inturrisi et al., 1988; Stachowiak et al., 1990) gene expressions, although the exact location of GRE in proENK and TH gene promoter/enhancer region has not been identified. As shown in Fig. 6A, CHX increased corticosterone levels in the adrenal gland in a time-dependent manner. Furthermore, the blood corticosterone level was also increased by CHX treatment, suggesting that the increase of corticosterone in the adrenal gland may be caused by the biosynthesis rather than blockade of secretion of corticosterone to blood. To examine the possible involvement of corticosterone in the regulation of CHX-induced proENK and TH mRNA expression, the effect of RU486 (a steroid receptor antagonist) on CHX-induced responses was studied. As shown in Fig. 6B, RU486 partially attenuated CHX-induced mRNA expression of proENK, but not TH. Although the selective inhibition of RU486 against CHX-induced proENK mRNA expression was not well understood, one explanation might be a difference in sensitivity of proENK and TH genes against glucocorticoid concentration. In cultured bovine adrenal medullary cells, nanomolar concentrations of dexamethasone were sufficient to increase the mRNA expression of proENK mRNA but not TH. TH mRNA was increased by a higher concentration (millimolar range) of dexamethasone (Stachowiak et al., 1990).

The positive role of glucocorticoids in the regulation of CHX-dependent proENK mRNA expression is supported by a previous study in rat adrenal glands from sham and hypophysectomized rats in which the basal level of enkephalin-containing peptide was higher in sham glands than in hypophysectomized glands. The dexamethasone-induced rise of enkephalin-containing peptide was higher in sham glands than in hypophysectomized rats in which the basal level of enkephalin-containing peptide was higher than in sham glands, which was well correlated with CRE DNA binding activity. Because CREB is known to alter the expression of specific target genes containing CRE and CRE-like DNA elements through the phosphorylation process rather than increasing its DNA binding activity (Gonzalez and Montminy, 1989; Arias et al., 1994), the increased phospho-CREB level may be enough to alter CRE-dependent gene expression without altering CRE DNA binding activity. Indeed, the supershift assay showed that CHX-induced phospho-CREB actively participated in the formation of CRE DNA binding activity. Therefore, our results suggest that CHX-dependent increase of the phospho-CREB level, rather than AP-1, seems to be involved in CHX-induced proENK and TH mRNA expression in the adrenal medulla. However, further study should be performed to determine whether CHX-induced phospho-CREB is enough for the activation of proENK and TH mRNA expression.

In conclusion, in this in vivo study, we have demonstrated for the first time that proENK and TH mRNA expression in the adrenal medulla can be up-regulated by pre-existing factors rather than newly synthesized transcriptional factors, such as AP-1 components. The adrenal medulla highly expresses basal AP-1 proteins, such as Fra-2, c-Jun, and JunB, but this seems to be unneeded for proENK and TH mRNA expression because CHX time dependently reduced these protein levels, but proENK and TH mRNA expressions were further increased. Although the exact reason for CHX-dependent increase of phospho-CREB levels is not currently understood, the phosphorylation of CREB is increased by CHX in a time-dependent manner, and phospho-CREB protein participates in the formation of CRE DNA binding complex. Therefore, our results suggest that CREB, rather than AP-1, may play a major role in the regulation of CHX-induced proENK and TH mRNA expression in the adrenal gland. Finally, although CHX increases plasma and adrenal gland corticosterone levels, the inhibitory effect of glucocorticoid receptor antagonist, RU486, was observed in CHX-induced mRNA expression of proENK, but not TH, suggesting that glucocorticoid is involved, at least partially, in the regulation of CHX-dependent proENK, but not TH, mRNA expression in the adrenal gland.

References
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