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REGULATION OF RAT ADRENAL MEDULLARY PHENYLETHANOL AMINE *N*-METHYLTRANSFERASE

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ABSTRACT: *Neural regulation of phenylethanolamine N-methyltransferase (PNMT) was studied with reserpine as a neuronal agent in rat adrenal medulla. The enzyme activity assay and northern blot analysis were performed to determine whether the induction of PNMT activity after reserpine treatment was associated with elevation of mRNA coding for PNMT. The i.p. administration of reserpine (2.5 mg/kg) on alternate days for 4 injections to rats brought about 30% increase of adrenal medullary PNMT activity and approximately 60% stimulation of the PNMT mRNA level in rat adrenal gland. A dose of 10 mg/kg of reserpine was chosen to perform optimum induction of PNMT activity in the rat adrenal gland based on the results of dose response curve of reserpine. Time course reserpine (10 mg/kg) effects on the rat adrenal medullary PNMT were as follows: 1. Peripheral PNMT activity reached maximum level after 7 days of drug treatment on alternate days. 2. Trans-synaptic stimulation by reserpine increased pretranslational activity of rat adrenal PNMT, but not translational activity. 3. Immunotitration of PNMT molecule after reserpine treatment indicated that reserpine produced an enzyme with greater antibody affinity than endogenous molecule in the rat adrenal gland.*

Keywords: *Phenylethanolamine N-methyltransferase, Reserpine, Catecholamine Synthesizing Enzyme, Rat Adrenal Medulla, Northern Blot, Immunotitration.*

INTRODUCTION

Phenylethanolamine N-methyltransferase (PNMT; EC 2.1.1.28; S-adenosyl-L-methionine: phenylethanolamine N-methyltransferase) is the final enzyme in the catecholamine biosynthetic pathway. It catalyzes the methylation of norepinephrine to epinephrine. The enzyme is enriched in the catecholamine-producing chromaffin cells of the adrenal medulla, where epinephrine serves as a hormone (Axelrod, 1962).

It has been previously stressed about the importance of neuronal and hormonal stimuli in the regulation of PNMT (Lima and Sourkes, 1986a; Bohn, 1983; Ciaranello, 1978; Ciaranello *et al.*, 1975; and Molinoff *et al.*, 1970), and other catecholamine synthetic enzyme, such as dopamine β-hydroxylase (DBH; Lima and Sourkes, 1986b; Ciaranello *et al.*, 1975) and tyrosine hydroxylase (TH; Mueller *et al.*, 1970) via distinct biochemical mechanisms. Glucocorticoids and other steroid hormones control steady state levels of the catecholamine synthesizing enzymes by modulating enzyme proteolysis (Wong and Ciaranello, 1982). In contrast, neuronal stimuli increase the activities of the enzymes above steady state values. Reflex activation of the splanchnic nerves to the adrenal medulla by drug such as reserpine or phenoxybenzamine effectively elevates the levels of the enzymes in the intact rats. Also, direct stimulation with acetylcholine or carbamylcholine increases the activity of the enzymes in the same manner (Ciaranello *et al.*, 1975). Previous studies of radioactive amino acid labeling have shown that the trans-synaptic induction of the enzymes is mediated by stimulation of *de novo* synthesis of the catecholamine synthetic enzymes without altering the proteolysis of the proteins.

We are interested in not only the induction of the adrenal medullary PNMT after the reserpine administration to the rat, but also the regulation of mRNA level coding for PNMT by reserpine. Since cDNA probe specific for PNMT mRNA is available to us, attempts have been made to determine whether reserpine regulates PNMT activity at the level of pretranslation or translation.

In this report, we describe the effect of reserpine to the adrenal medullary PNMT, neuronal regulation of PNMT-specific mRNA and immunotitration studies for the PNMT molecule. Northern blot analysis showed that the rat adrenal medullary PNMT is regulated at the pretranslational level by reserpine as a neuronal agent. The preliminary results of immunotitration of adrenal extracts with anti-PNMT antiserum suggested that reserpine treatment leads to an enzyme with increased antibody affinity.

MATERIALS AND METHODS

Tissue Preparation

Male Sprague-Dawley rats weighing 150-180 g initially were used in all experiments. They were obtained from Simonsen, Gilroy, CA, USA. Animals were maintained in a facility with automatic temperature and lighting regulation (light-dark cycle of 12 hours), and free to food (animal lab chow) and tap water. Follwoing one week of housing acclimitization, reserpine (Serpasil, Ciba-Geigy Phamaceutical Company, Summit, NJ, USA) was administered to an experimental group of rats by intraperitoneally (i.p.). Reserpine was dissolved in the vehicle which was recommended by Ciba-Geigy. The drug dosages and number of injections are fully described in the text. Experimental groups of animals were sacrificed 24 hours after the last injection and control animals were sacrificed at the same time of those of the experimental groups by cervical dislocation. Adrenal glands were excised and cleaned of fat. Meduallry tissue was obtained by nicking the cortical capsule and gently squeezing out the medulla. Tissues were frozen immediately at -85°C until used.

Enzyme Assay

A pair of adrenal medulla was homogenized in 5 volumes of 50 mM Tris-HCl buffer, pH 7.4 containing 0.2% bovine serum albumin and 0.2% Triton X-100 (Sigma, St. Louis, MO, USA) for 15 seconds using a Heat Systems Ultrasonics cell disruptor at a setting of 4 (Heat Systems Ultrasonics, Plainview, NY USA).The homogenates were centrifuged for 2.5 minutes at 10,000 × g in a Beckman microfuge, Model B (Beckman Instruments, Inc., Palo Alto, CA, USA) to remove cellular debris. The supernatants were dialyzed for 1 hour at 4 °C against 50 mM Tris-HCl, pH 7.4 with several changes and then diluted to a final volume of 5 m*l* using the same buffer. PNMT activity was assayed using 50 μl alliquots of the supernatants as described by Wong *et al.*, (1982). Phenylethanolamine (Sigma, St. Louis, MO, USA) and S-adenosyl-[^{3}H-methyl]-methionine (New England Nuclear, Boston, MA, USA) were used as a substrate and a methyl donor, respectively, for the enzyme assay.

Northern Blot Analysis

Total RNA isolated from tissues by the modified method of Chirgwin et al. (1979) as described earlier (Davis *et al.*, 1986). A pair of adrenal medulla was homogenized in guanidine isothiocyanate lysis solution containing 4M guanidine isothiocyanate (International Biotechnologies, Inc., New Haven, CT, USA), 25 mM sodium acetate, pH 6.8 and 8% β-mercaptoethanol. The homogenates were layered over a cushion of 5.7 M CsCl, 25 mM sodium acetate, pH 6.0. RNAs were fractionated by ultracentrifugation in an SW-55 rotor at 35,000 rpm for 21 hours at 20 °C (Gilsin *et al.*, 1974) or in an TL-100 rotor (Beckman Instruments, Inc., Palo Alto, CA, USA) at 43,000 rpm for 18 hours at 20 °C (Mornex *et al.*, 1986). Poly (A)$^+$-enriched mRNA was isolated by chromatography on oligo-dT cellulose.

PNMT specific cDNA probe (courtesy of Dr. Berry Kaplan, Pittsburgh University School of Medicine) was excised from pBR 322 cloning vector with Pst 1 restriction endonuclease, then labeled with [α-^{32}P] dCTP (New England Nuclear, Boston, MA, USA) using the random primed DNA labeling method of Feinberg and Vogelstein (1984).

RNAs were denatured with 0.66M formaldehyde, electrophoresed on a horizontal 1% agarose gel, transferred to nitrocellulose membrane (Schleicher & Schuell) and then hybridized with radiolabeled cDNA probe. The autoradiograph was obtained by placing the filter in a cassette with an intensifying screen and Kodak XAR-5 film for 24-48 hours at -70 °C.

Immunotitration of PNMT

The same tissue homogenates of rat adrenal medulla were used for the titration studies as for the enzyme assay. A male New Zealand rabbit (Simonsen, Gilroy, CA, USA) was immunized with purified bovine PNMT as described by Ciaranello *et al.*, (1975). The purification of bovine adrenal medullary PNMT with a greater homogeneity was performed as privously described (Wong *et al.*, 1986). The antiserum was stored at -70°C until used for immunotitration of PNMT from rat adrenal medullary supernatants.

RESULTS

Effects of Reserpine on the PNMT Activity of the Rat Adrenal Medulla

We examined the changes in the rat adrenal medullary PNMT activity by splanchnic innervtion using reserpine. Reserpine, at a dose of 2.5 mg/kg of body weight, was administered on alternate days for a total of four injection by i.p. Two groups of animals (six per group), one group for control and the other for experimental, were examined. Animals were sacrificed 24 hours after the last injection. Tissue preparation and the assay of enzyme activity were performed as described in the Materials and Methods. The enzyme activity was expressed as units per pair of adrenal medulla, where 1 unit was the formation of 1 nanomole of product per hour. The values were expressed as mean ± standard error of mean. The PNMT activity was increased in the rat adrenal medulla after reserpine treatment as previously shown (Ciaranello and Black, 1971). The control value was 6.7 ± 0.1 and the experimental values, 8.5 ± 0.2 units/pair of ad. Therefore, initial dose of 2.5 mg/kg of reserpine causes 30% induction of rat adrenal PNMT activity.

Effects of Reserpine on the Messenger RNA Coding for PNMT

We further attempted to understand how splanchnic innervation mediates PNMT stimulation *in vivo*. To determine whether the induction of PNMT activity after the administration of reserpine was associated with elevation of mRNA coding for PNMT, northern blot analysis was performed on total RNA and Poly $(A)^+$ RNA obtained from rat adrenal medulla. For the group of reserpine treatment, rats (twelve animals) were administered with 2.5 mg/kg of reserpine, every other day for four injection i.p. Animals (control and experimental group) were killed 24 hours after the last injection. The adrenal medulla tissues were pooled for the total RNA preparations.

Fig. 1 depicts autoradiogram of northern blot hybridization of bovine PNMT cDNA to rat mRNA. The size of PNMT mRNA and intensity of hybridization to the bovine PNMT cDNA under conditions of high stringency of washing as described in the legend of Fig. 1, show the authentisity of PNMT mRNA in the rat adrenal. Furthermore, there was no hybridization of the cDNA probe to poly $(A)^+$ RNAs from liver (lane L) where the PNMT is not expressed.

More importantly, this autoradiogram in Fig. 1 clearly demonstrates the difference in the intensity of the hybridization between control and reserpine treated rats. When comparing the lane 3 with 4 (total RNA hybridization) and lane 7 with 8 (poly $(A)^+$ RNA hybridization), it is apparent that the reserpine causes a significant induction of the PNMT mRNA in the rat adrenal. Densitometric laser scanning of the autoradiogram reveals approximately 60% induction of the PNMT message in both total RNA and poly $(A)^+$ RNA hybridization (Data not shown).

Therefore, the northern blot analysis evidently indicates that the regulation of the rat adrenal medullary PNMT by reserpine as a neuronal stimulus occurs at the pretranslational level.

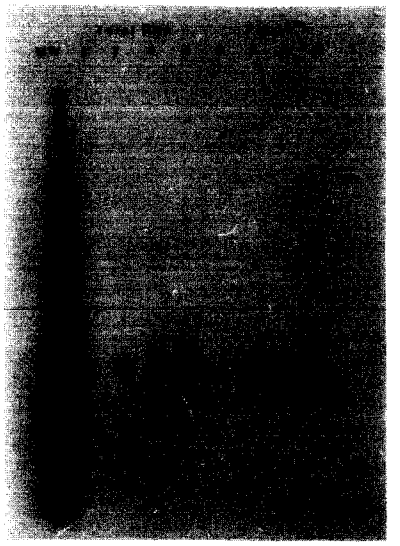

Fig. 1. Neuronal effects in PNMT mRNA in the rat adrenal medulla. Total RNA and poly $(A)^+$ RNA from control and reserpine treated rats were fractionated by electrophoresis on formaldehyde denaturing gels then transferred to nitrocellulose. Approximately 3.0×10^7 cpm of ^{32}P-labelled PNMT specific cDNA probe (spun-column purified from unincorporated radioisotopes; specific activity of 1.5×10^9 cpm/μg) were denatured with 1M NaOH and neutralized with Tris-HCl buffer, pH 7.4 and 1M HCl. The denatured cDNA probe was then added to hybridization buffer containing 10% Dextran sulfate, 40% formamide (deioized), 4 × SSC, 20 mM Tris-HCl, pH 7.4, 1 × Denhardt's solution, and 20 μg/ml of salmon sperm DNA. Following hybridization with radiolabeled probe for 48 hours at 42°C, the filter was washed in 2 ± SSC and 0.1% SDS at room temperature for 5 minutes and replaced with fresh buffer three more times at 20 minutes intervals. Then, the final wash of the filter was two 20 minutes washes in 0.1 × SSC and 0.1% SDS at 65°C. The filter was exposed to X-ray film at -70°C with an intensifying screen for 24-48 hours. ^{32}P-labeled RNA ladders (BRL, Gaithersburg, MD, USA) were used for molecular weight standard (MW). Lane 1,2 and 3 represent 6.0, 10.5 and 15 μg of total RNA obtained from the control rat adrenal medulla on the gel, respectively. Lane 4 shows 15 μg of total RNA from the reserpine treated rats, lane 5, 6 and 7 represent 0.7, 1.4 and 2.1 μg of poly $(A)^+$ RNA from the control, respectively, and lane 8 shows 2.1 μg of Poly $(A)^+$ RNA from the experimental rats. Lane L represents 5.0 μg of poly $(A)^+$ RNA from normal rat liver.

Reserpine Dose Response Curve for PNMT Activity

Dose response curve of reserpine was performed to determine the optimum dose to increase PNMT activity both in the adrenal gland and in the brain (Pons and Medulla).

Low doses of reserpine ranging from 2.0 to 5.0 mg/kg of the drug were injected to animals for four times on the alternate days. The peripheral enzyme activity for the low dose reserpine treatment was shown in Table 1. The PNMT activity was expressed as units per pair of adrenal medulla and the values were expressed as mean ± standard error of mean.

Then, we examined the high dose of the drug up 10 mg/kg of body weight (Table 2). The method of the drug treatment was as same as that of low dosage reserpine experiment. Based on the results of dose response curve experiments, 10 mg/kg of

Table 1. Reserpine dose (low) response to rat adrenal medullary PNMT activity

Reserpine Dose mg/kg	n	PNMT Activity	
		units/pair of ad.*	fold change
0	9	7.9±0.1	1.0
2.0	8	12.2±0.4	1.5
2.5	8	10.6±0.4	1.3
3.0	8	11.9±0.5	1.5
4.0	8	12.3±0.4	1.6
5.0	8	13.4±0.3	1.7

Table 2. Reserpine dose (high) response to rat adrenal medullary PNMT activity

Reserpine Dose mg/kg	n	PNMT Activity	
		units/pair of ad.*	fold change
0	7	7.5±0.6	1.0
2.5	6	10.7±0.2	1.4
5.0	6	12.9±1.3	1.7
7.5	6	14.7±0.9	2.0
10.0	6	15.4±1.0	2.1

*Mean ± S.E.M.

reserpine, a dose which optimally increases the enzyme activity both in periphery and CNS (Data not show), was chosen hereafter.

Time Course for the Effects of Reserpine on Adrenal Medullary PNMT.

We next examined the effects of reserpine at the different time point on the overall regulation of rat adrenal medullary PNMT, including the enzymatic activity, mRNA for PNMT and immunotitration of the enzyme molecules. Rats were treated with 10 mg/kg of reserpine, a dose which optimally increases PNMT activity. At this dose, peripheral enzyme activity reached maximum levels after 7 days of drug on alternate days. As demonstrated in Table 3, the enzyme activity was increased in a linear fashion, such as 1.1, 1.3, 1.6 and 1.9-fold after 1, 2, 3 and 4 injections of the drug, respectively. In contrast, a sigmoidal change in the level of mRNA for PNMT was observed in the adrenal gland (Table 3). Extent of induction of enzyme activity and mRNA after 4 injections of the drug, however, showed quite similar. Thus, the results of such experiments suggested that reserpine treatment increases pretranslational activity but not translational activity for rat adrenal PNMT.

Immunotitration of PNMT was performed to determine whether the newly synthesized PNMT molecule by reserpine showed the same affinity to the PNMT specific antibody as endogenous PMNT molecule in the adrenal gland, as shown in Table 3. The same amounts of adrenal extracts from each group of rats were used for the titration study, which enabled us to compare the equivalence point of each group. Fold changes of equivalence point upon time course treatment of the drug showed smaller increment than that of the enzyme activity. This result of the titration study implied that reserpine treatment produces an enzyme with greater antibody affinity.

Table 3. *Time course reserpine effects of PNMT activity, mRNA coding for PNMT, and immunotitration of rat adrenal medullary PNMT molecule*

Number of Injection	PNMT Activity		Northern	Immunotitration	
	Units/pair of ad.*	Fold change	*Fold change	Equivalence point*	Fold change
0	6.4±0.3	1.0	1.0	8.4±0.2	1.0
1	7.2±0.3	1.1	1.6	9.0±0.1	1.1
2	8.6±0.3	1.3	1.7	10.1±0.2	1.2
3	10.1±0.4	1.6	1.6	12.1±0.3	1.4
4	12.0±0.6	1.9	2.0	11.5±0.3	1.4

*Mean ± S.E.M.

DISCUSSION

In this report, we have described the neuronal regulation of the rat adrenal medullary PNMT. The enzyme activity was increased by 30% with 4 injections of the reserpine (2.5 mg/kg) on alternate days. Lima and Sourkes (1986 a) and Ciaranello and Black (1971) showed the similar induction of PNMT with comparable dose of reserpine. However, Ciaranello *et al.*, (1975) demonstrated that much greater induction of PNMT was produced through the treatment similar to our experiments. Although same strain of rats was used for those experiments, there were some differences in terms of nutrition as well as environment of animals that were used for those experiments. Moreover, the purity and quality of the drug might be vary.

Although, the molecular cloning of cDNA as well as the structure of the gene for bovine and human PNMT have been well characterized by many researchers (Batge *et al.*, 1988; Batter *et al.*, 1988; Kaneda *et al.*, 1988; Weisberg *et al.*, 1988; Batge *et al.*, 1986), investigations for the rat PNMT have been suprisingly rare. In our experiments, as others did (Evinger *et al.*, 1986), bovine PNMT cDNA probe was used to hybridize the rat mRNA in the northern blot analysis. Our research resulted that not only the cross-reactivity, but also the specificity to the PNMT mRNA were significantly high. 5 ug of poly $(A)^+$ RNA extracted from liver, which is 2.5-5times in excess than common use, was introduced to the lane of negative control. Result was not reaction at all in that lane.

Since the radiolabeled MW standard was overexposed, we used the ribosomal RNA in the gel as a size marker to determine the size of rat adrenal PNMT mRNA, which turned out to be approximately 1.1-1.2 Kb in length.

In conclusion, our research results suggest that the adrenal medullary PNMT activity is regulated by the reserpine, primarily at the level of pretranslation. Also, compared to the control group, the reserpine treated group showed that the reserpine might cause the antigenic determinants in the PNMT more exposed.

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