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Tyrosine Hydroxylase Activity and mRNA in Rat Locus Coeruleus and Adrenals Following Chronic Ethanol Treatment and Acute Cold Stress

YongKyu Lee* and DongHa Park[1]

Department of Food Engineering, Dongseo University, Pusan 616-010, Korea
[1]Department of Molecular Neurobiology, Cornell Medical College, The Burke Rehabilitation Center, 785 Mammaroneck Ave., White Plains, NY 10605, USA
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Abstract: Sprague-Dawley male rats (150 g) were chronically treated with 5 v/v % ethanol admixed with nutritionally complete liquid diet and fed ad libitum for 3 weeks. Controls were pair fed with the isocaloric sucrose liquid diet. One half of each group was exposed to cold stress at 4℃ either for 24 h (for determination of mRNA by in situ hybridization) or for 48 h (for determination of enzyme activity). Chronic ethanol treatment (ethanol) did not affect tyrosine hydroxylase (TH) mRNA level in locus coeruleus (LC) of brain and adrenal medulla (AM) compared to controls. Cold stress showed strong increase of TH mRNA level in LC and AM compared to controls. Pretreated ethanol reduced the increased TH mRNA level by cold stress in LC and AM. Ethanol did not affect TH activity in LC and adrenal glands (adrenals). Cold stress increased TH activity in LC but not in adrenals. Pretreated ethanol did not reduce the increased TH activity by cold stress in LC but this result was not shown in adrenals. It is suggested that ethanol does not affect the message level and enzyme protein level for TH in LC and AM in normal rat. It is also hypothesized that pretreated ethanol reduces the magnitude of acute cold stress response, that is induction of TH mRNA in LC and AM, and does not reduce the increased TH enzyme protein that is also acute cold stress response in LC.

Key words: cold stress, ethanol, tyrosine hydroxylase activity, tyrosine hydroxylase mRNA.

Varying results have been obtained on the effect of chronic ethanol treatment (ethanol) on catecholamine levels in brain ever since Gursey and Olson (1960), and they are either a lack of effect or an elevation of catecholamine level according to the duration of treatment. But few reports have been obtained on the effect of ethanol on the catecholaminergic enzyme system. Carlsson *et al.* (1973) reported that TH activity of whole brain was increased by a single dose of ethanol and Detering *et al.* (1980) reported that TH activity of whole brain was increased in offspring of rats fed a diet containing ethanol.

There are a few reports (Lehrer *et al.*, 1974; Levenson *et al.*, 1980; Sher *et al.*, 1982) that chronic ethanol consumption reduces the magnitude of response to stress in human or rat, occassionally that is said either tension reduction by lower doses of ethanol or stress response dampening by higher doses of ethanol. In those reports, ethanol reduced simple physiological or psychyatric responses to laboratory stressors such as loud tones, high affect words, shaking and electrical stimuli. Those responses are pulse transmission, cardiac and electrothermal responses reported in 1980s. Biochemical response change to stress was reported (Thoenen, 1970; Chuang *et al.*, 1974; Fluharty *et al.*, 1985; Tank *et al.*, 1985), that was TH activity increase by cold stress. TH mRNA increase by cold stress was also reported (Stachowiak *et al.*, 1985; Tank *et al.*, 1985; Stachowiak *et al.*, 1986).

In this study, we aimed to investigate ethanol effect on TH enzyme system either in normal rat or in cold stressed rat. According to the preliminary test in our laboratory, ethanol did not affect significantly TH activity in LC and adrenals (these data were not shown in this paper), therefore we investigated how ethanol affected the cold stress responses. First, we investigated the effect of ethanol on TH activity and TH mRNA level in normal rat, and secondly we investigated the effect of ethanol on TH activity and mRNA level in acute cold stressed rat.

*To whom correspondence should be addressed.
Tel: 82-51-320-1585, Fax: 82-51-312-2389.
E-mail: lyk@kowon.dongseo.ac.kr

Materials and Methods

Chromic ethanol treatment

Sprague-Dawley male rats (Charles River, Wilmington, MA) weighing 150 g were housed in individual shoe box cages bedded with shavings. One week after arrival, the animals were paired according to the proximity of their body weights. One of each pair was assigned to the ethanol group and the other served as its pair-fed control. During the experiments, the sole source of diet and water was nutritionally complete liquid diet (Bio-serve, Liquid-Rat Diet, Lieber and DeCarli, 1982).

Ethanol was given *ad libitum*, admixed in liquid diet to make 5 v/v% final concentration. The control animals received the liquid diet containing sucrose that was made isocaloric to the 5 v/v% ethanol diet and pair-fed. The volume of liquid diet consumed and the body weight were measured and recorded daily. The amount of ethanol consumed was calculated and expressed as amount consumed per kg of body weight of the animal per day. The total duration of the treatment was 3 weeks.

Stress condition

One half of the ethanol group and its pair-fed animals were randomly selected and exposed to cold stress at 4℃ either for 24 h (for determination of mRNA by in situ hybridization) or for 48 h (for determination of enzyme activity). The feeding of animals was continued as is described above.

Measurement of TH activity

The animal was sacrificed by decapitation. LC of brain and adrenals were dissected bilaterally and homogenized each in 400 μl, 500 μl, and 2 ml of 0.5 mM Tris-Cl buffer (pH 7.5) containing 0.1% Triton X-100, respectively. Homogenates were centrifuged at 10,000×*g* for 30 min at 0℃. Supernatant was used for the measurement of TH activity. TH activity was determined by Park *et al.* (1990)'s method. The incubation mixture (in μmol/0.5 ml total volume): sodium acetate buffer (pH 6.1), 100.0; ferrous sulfate, 0.5; 6.7-dimethyl-5,6,7,8-tetrahydropterine ($DMPH_4$), 1.0; mercapto ethanol, 20.0; sodium phosphate, 1.0; L-tyrosine-[1-^{14}C], 0.05 (10 μci/μmol, 1.1×10 dpm); a crude kidney aromatic L-aminoacid decarboxylase preparation, 7.5 U (nmol/30 min); and pyridoxal phosphate, 0.005. The enzymatic reaction was carried out at 37℃ for 30 min. The reaction was stopped by adding 0.5 ml of 10% trichloric acid. The radioactive CO_2 liberated from the L-DOPA-[1-^{14}C] formed from tyrosine-[1-^{14}C] by TH, was absorbed on a filter paper wetted with NCS solubilizer (Du Pont-New England Nuclear) and measured as described in the assay of dopamine decarboxylase based on $^{14}CO_2$ evolution from [carboxy-^{14}C]-dopamine. Specific activity was defined as pmole of dopamine formed/mg protein/min. Protein concentration was determined by the method of Lowry *et al.* (1951).

In situ hybridization

Rat were anesthetized with pentobarbital (50 mg/kg) intraperitoneally and then rapidly perfused transcardially with 0.9% sodium chloride, containing 0.5% sodium nitrite and 1,000 U heparin/100 ml. This was followed by a slow perfusion with ice-cold 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Brains and adrenals were immediately removed, cut into blocks, and submerged in the ice-cold fixative where they remained for 1 h. The blocks were then rinsed twice with phosphate buffer and cryoprotected by storing the tissue in 30% sucrose overnight at 4℃. Tissue sections of 30 μm thickness were cut on a sliding microtome and stored in 20 ml glass vials filled with 2×SSC (0.3 M sodium chloride/0.03 M sodium citrate, pH 7.0) with 10 mM Dithiothreitol (DTT) at 4℃. Hybridization method was determined by a minor modification of the method of Valentino *et al.* (1987). This storage solution was then replaced with prehybridization buffer containing 50% formaldehyde, 10% dextran sulfate, 2×SSC, 1×Denhart's solution, 50 mM DTT, and DNA. Prehybridization was carried out for 1 h at 48℃. A 0.4 kb *Kpn*I-*Eco*RI restriction fragment of rat TH cDNA (Carroll *et al.*, 1991), labeled with [^{35}S]dCTP by random priming, was added to each vial containing tissue sections as a probe. Hybridization was carried out overnight at 48℃. After extensive washes in decreasing concentrations of SSC, 10 min steps of 1:1 dilutions starting at 2×SSC and ending with 0.1×SSC, tissue sections were mounted onto gelatin-subbed slides, air-dried and dehydrated through graded ethanols (70, 90, 100 %). Finally the slides were dipped into undiluted Kodak (Rochester, USA) NTB-2 in the darkroom. After storage in light-light boxes at 4℃ for 7 to 21 days, each slide was developed in Kodak D-19 developer for 2 min and fixed for 2×4 min punctually to get a same conditioned result for each experimental group. Sections were counterstained with cresyl violet and cover-slipped with Permount (Fisher Scientific, Springfield, USA).

Statistical analysis

Statistical analysis of the data was performed using student's t-test compared to control rat. Only the ethanol plus cold stressed group was analyzed by two way ANOVA test between cold stressed group and ethanol group.

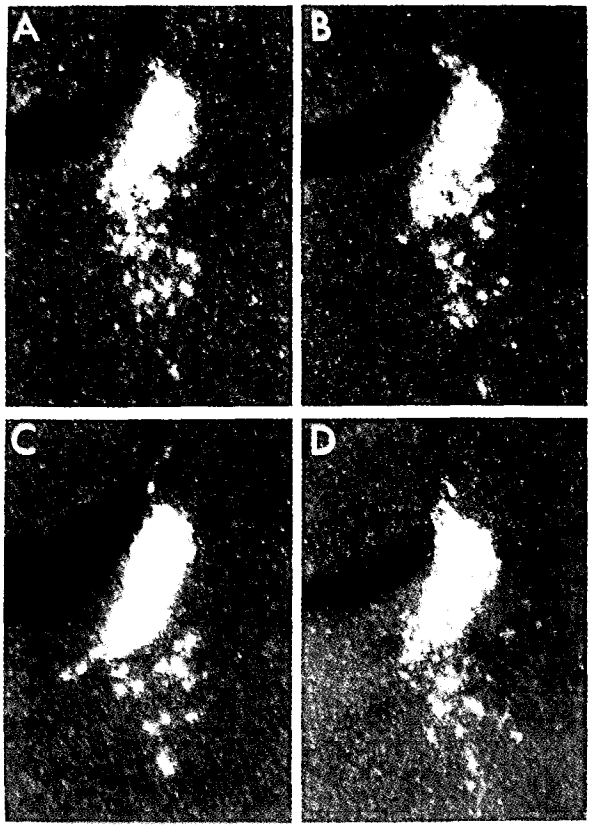

Fig. 1. *In situ* hybridization to TH mRNA in LC. Dark-field photomicrographs illustrate hybridization signals for TH message of isocaloric sucrose fed control animals (A), those chronically treated with ethanol (B), those acutely exposed to cold (C), and those chronically treated with ethanol followed by acute exposure to cold (D). Note that cold stress leads to strong induction of TH mRNA in LC but ethanol treatment appears to reduce the increased TH mRNA levels by cold stress in LC. In contrast, ethanol treatment alone do not show any change in grain density. Bar: 200 μm.

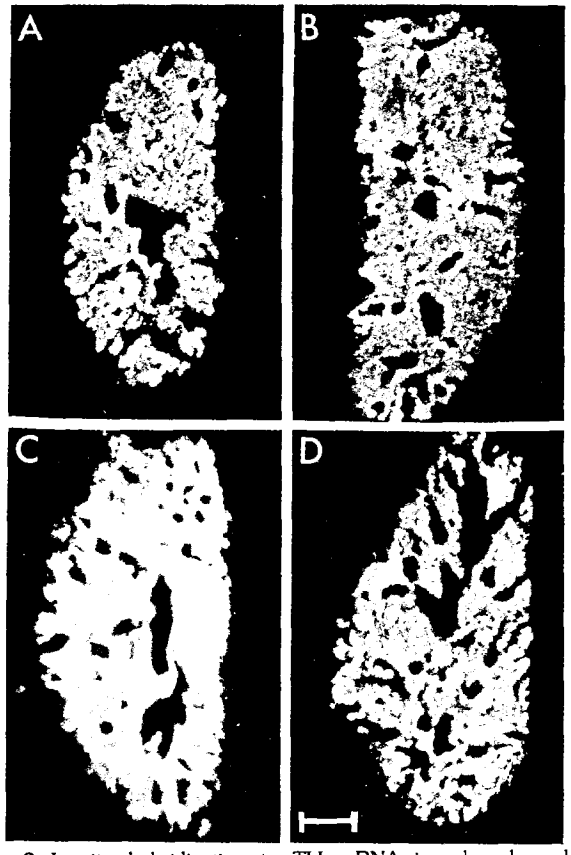

Fig. 2. In situ hybridization to TH mRNA in adrenal medulla (AM) of the rat. Dark-field photographs illustrate hybridization signals for TH message of isocaloric sucrose fed control animals (A), those chronically treated with ethanol (B), those acutely exposed to cold (C), and those chronically treated with ethanol followed by acute expososure to cold (D). Note that ethanol treatment alone do not cause any alteration in message levels, but cold stress also induces strong response in the message levels similar to the observation made in LC. Ethanol also reduce the increased TH mRNA levels in AM like the LC. Bar: 300 μm.

Results

TH mRNA

Fig. 1. shows that cold stress lead to strong induction of TH mRNA in LC but pretreated ethanol appeared to reduce the increased TH mRNA by cold stress in LC. In contrast, ethanol alone did not show any change in TH mRNA level.

The changes of TH mRNA level resulting from ethanol and cold stress in adrenal medulla are shown in Fig. 2. Ethanol alone did not cause any alteration in message levels, but cold stress increased TH mRNA level similar to the observation made in LC. Pretreated ethanol also reduced the increased TH mRNA by cold stress in AM like the LC.

Enzyme activity

Table 1 summarizes the effect of ethanol in combination with acute cold stress on TH activity of rat LC and adrenals. TH activities of ethanol group in LC and adrenals were not changed significantly compared to control group. TH activity of cold stressed group signifi-

Table 1. Differential effects of ethanol in combination with acute cold stress on tyrosine hydroxylase (TH) activity of rat locus coeruleus and adrenals

Treatment	Tyrosine hydroxylase activity	
	Locus coeruleus	Adrenal glands
Control	0.164± 0.0187 (5)	6.69± 0.279 (5)
Ethanol	0.152± 0.0128 (5)	6.40± 0.145 (5)
Cold stress	0.253± 0.0289[a] (5)	6.84± 0.273 (5)
Ethanol+Cold stress	0.394± 0.0467[b] (5)	6.57± 0.816 (5)

TH activity (mean± SEM) is expressed in nmol/mg protein/15 min at 30℃. Number of animals used is given in parenthesis.
[a] $p<0.05$ when compared to control.
[b] $p<0.05$ when compared to ethanol and cold stress by two way ANOVA test.

cantly increased compared to control group only in LC. TH activity of ethanol plus cold stressed group significantly increased compared to cold stressed group and compared to ethanol group, but this increasement was not shown in adrenals.

Discussion

In this work, we tested ethanol effect on TH in LC and adrenals in normal rat and cold stressed rat. LC is a compact nucleus consisting almost entirly of the cell bodies of catecholaminergic neurons in central nervous system (Dahlstrom *et al.*, 1964; Ungerstedt *et al.*, 1971; Astone-Jone *et al.*, 1984), and adrenals have catecholaminergic neurons pheripherally. TH is the rate-limiting enzyme in the catecholamine biosynthetic pathway, and steady-state levels of catecholamines, namely, dopamine, norepinephrine and epinephrine depend on TH activity.

Fig. 1 and 2 show that ethanol did not change TH mRNA level in LC and AM and cold stress lead to strong induction of TH mRNA in both LC and AM of normal rat, but ethanol reduced the increased TH mRNA level by cold stress in both LC and AM. Our result that one day cold stress lead to strong induction of TH mRNA in LC and AM agrees with the other results (Tank *et al.*, 1985). It is considered that ethanol alone does not affect TH mRNA level in normal rat, but pretreated ethanol suppresses the cold stress response that is strong induction of TH mRNA in both LC and AM.

From the reports (Stachowiak *et al.*, 1985; Tank *et al.*, 1985; Stachowiak *et al.*, 1986) that enzyme activity increase is preceded by $mRNA^{TH}$ level increase, we investigated the TH activity both in LC and adrenals. From the result shown in Table 1, it is suggested that ethanol does not affect TH activity both in LC and adrenals. It is considered that TH activity change was not shown because of being not preceeded by TH mRNA change by ethanol (shown in Figs. 1 and 2).

From the results shown in Fig. 1 and Table 1, TH activity of ethanol plus cold stressed group was increased about 50% more than that of cold stressed group in LC (Table 1), though TH mRNA level was decreased compared to cold stressed group (Fig. 1). It is considered that the reason for this discrepancy between TH mRNA level and TH activity of ethanol plus cold stressed group may be due to a duration of cold stress. We measured mRNA level after one day cold stress and enzyme activity after two days cold stress, each stress is acute. It is considered that mRNA level of two days cold stressed group can be increased more than that of one day cold stressed group, so enzyme activity may not be decreased by pretreated ethanol. It is also considered that two days cold stress can be in the middle of maximum increase of TH mRNA and TH activity by cold stress. Fluharty *et al.* (1985) got a maximal TH activity increase about 4 fold in adrenals by a chronic cold stress for more than 6 days.

Immunochemical studies demonstrated that the increased TH activity reflected an increased amount of enzyme protein (Hoeldtke *et al.*, 1974) and suggested that increase in adrenal TH activity was mediated by an increase in intracellular enzyme protein concentration (Joh *et al.*, 1973; Reis *et al.*, 1974; Tank *et al.*, 1985). From those suggestions, it is hypothesized that ethanol pretreatment does not supress the increased TH enzyme protein amount by cold stress in LC from the result that ethanol pretreatment did not reduce the increased TH activity by cold stress in LC.

From the results shown in Fig. 1, 2 and Table 1, cold stress increased TH mRNA level and TH activity in LC, but cold stress increased only TH mRNA level without accompanying TH activity increase in adrenals. From these discordant results in TH mRNA and activity between LC and adrenals, it is considered that LC can respond more sensitively than adrenals to two days cold stress because LC is a more compact nucleus consisting almost entirly of the cell bodies of catecholaminergic neurons than adrenals (Fuxe *et al.*, 1978; Moore and Bloom, 1978; 1979).

In conclusion, the results from this study indicate that ethanol does not affect the transcription of the gene for TH both in LC and AM and subsequently does not affect the TH enzyme protein level in normal rat. It is also indicated that ethanol pretreatment reduces the increased transcription level for TH by acute cold stress both in LC and AM, and does not reduce the increased protein level for TH by cold stress in LC. More investigation about the effect of pretreated ethanol on TH enzyme protein is needed with chronic cold stress.

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