Pergamon

Pharmacology Biochemistry and Behavior, Vol. 51, No. 4, pp. 775–780, 1995

0091-3057/95 $9.50 + .00

0091-3057(95)00030-5

Tyrosine Hydroxylase Expression in Rat Adrenal Medulla: Influence of Age and Cold

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Received 5 August 1994

TÜMER, N. AND J. S. LAROCHELLE. *Tyrosine hydroxylase expression in rat adrenal medulla: Influence of age and cold.* PHARMACOL BIOCHEM BEHAV **51**(4) 775–780, 1995. – Chronic and cold exposure is associated with an increase in adrenal medullary tyrosine hydroxylase (TH) activity and expression that may be important for the regulatory response to cold. Senescent rats do not maintain their body temperature as well as young rats. We investigated the ability of the catecholaminergic system of older rats to respond to cold stimulus. TH activity, TH immunoreactivity, and TH mRNA were assessed in adrenal medullae of male F-344 rats of 3 and 24 months of age following 48 h of mild (8°C) cold exposure. In control rats, basal levels of TH activity were increased by 2.9-fold, TH immunoreactivity by 1.3-fold, and TH mRNA by 2.3-fold with age. In the young rats there were increases after a 48-h cold exposure in TH activity, TH immunoreactivity, and TH mRNA per pair of adrenal medullae. In contrast, in senescent rats there were no significant changes in these parameters following cold exposure. These data suggest that the induction of TH activity is impaired in senescent rats following cold exposure and that there is a loss of plasticity with respect to the TH gene expression.

F-344 rats Cold exposure Tyrosine hydroxylase

ONE OF THE most serious consequences of the aging process is an inability to respond to environmental stimuli. Physiological and pathologic alterations with aging are associated with changes in adrenergic neurotransmission in the peripheral as well as in the central nervous system. For example, using foot shock or 2-deoxyglucose as a stimulus for the adrenergic neurohumoral axis, it has been shown that circulating levels of norepinephrine did not increase as much in old as in young rats (20). In addition, with aging, circulating catecholamines (CAs) are elevated in both humans and laboratory animals at rest (6,10,14,15,33). These elevations in circulating CAs may be related to the increased release of CAs from sympathetic ganglia and adrenals (2,15,24). The latter may be the result of the progressive increase in the synthesis of both epinephrine and norepinephrine content with age (24). Parallel to these changes we, as well as others, have reported that tyrosine hydroxylase (TH) activity and TH mRNA increase with age in the rat adrenal medullae (16,31–33).

TH is generally regarded as the rate-limiting step in the biosynthesis of CAs, and its activity is an important regulatory step in this pathway (22). Cold exposure is known to enhance the synthesis and release of CAs in the peripheral as well as the central nervous system, including the brain, adrenal medulla, and heart (11,12,17,19,34). Furthermore, previous reports have suggested that the elevated TH mRNA levels after cold exposure in the brain stem and adrenal medulla mediate the increases in TH activity induced by exposure to the cold (3,27,28). These elevations in sympathetic activity in response to cold exposure may be an important factor in mediating the thermogenic and heat retention processes that maintain body temperature. Older rats, however, do not maintain their body temperature as well as young rats when exposed to cold (21), possibly due to an inadequate response to catecholamine systems.

We investigated the ability of the catecholaminergic system of older rats to respond to cold stimulus. TH activity, TH

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immunoreactivity, and TH mRNA were assessed in adrenal medullae of male F-344 rats of 3 and 24 months of age following 48 h of mild (8°C) cold exposure.

METHOD

Animals

Fischer-344 (F-344) male rats, 3 and 24 months old, were obtained from a colony maintained for the National Institute on Aging at Harlan–Sprague Dawley Labs., Inc. (Indianapolis, IN) under contract with the National Institute on Aging. On arrival, rats were examined and remained in quarantine for 1 week. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals. Rats were housed individually in microisolated cages and maintained on Purina Rat Chow ad lib with a 12L : 12D cycle (0600 to 1800 h). Experiments were started 60–90 min after the beginning of the light cycle. Food and water were provided during the overnight periods of cold exposure, and bedding material was kept at an absolute minimum. Ambient temperature was 26°C. The median life span of F-344 rats is 27 months (9).

Cold Exposure

Rats were maintained at room temperature (26°C) or exposed to the cold (8°C) for 1 h or 48 h. For cold exposure, animals were placed in a Revco Cryo-frig (REC 230, 4-A-N-O) with circulating air supply, one animal per cage. Control animals, one per cage, were maintained in the same room but outside of the Cryo-frig. We chose a temperature of 8°C for cold exposure based on our preliminary studies that indicated two of four aged rats did not survive 5°C temperature.

Body temperature was monitored in F-344 rats by telemetry as described previously (25).

Tissue Preparation

Animals were deeply anesthetized with pentobarbital, and adrenal glands were removed quickly and immediately frozen by immersion in liquid nitrogen. Tissues were stored at −80°C. At the time of assay, adrenal glands were decapsulated and medullae separated from the cortex. To insure the recovery of all the medullary tissue, some cortical tissue may have been included. Adrenal preparations were weighed and homogenized in 100 μl of phosphate buffer (2 mM $NaPO_4$, 0.2% Triton, pH 7.0). Protein was determined by the method of Bradford (4).

Tyrosine Hydroxylase Activity

TH activity was measured using a modification of the radioenzymatic assay as described by Reinhard et al. (23). Briefly, 25 μl of homogenate was analyzed at pH 7.0 in the presence of 6-MPH_4 (1.5 mM) and [3,5-^{3}H]tyrosine (100 μM; 1 μCi/reaction), in a total volume of 50 μl for 15 min at 37°C. The assay is based upon the release of ^3H_2O from [3,5-^{3}H]-L-tyrosine with adsorption of the isotopic substrate (and its metabolites) by an aqueous slurry of activated charcoal. Unbound ^3H_2O was analyzed by liquid scintillation spectrometry.

Tyrosine Hydroxylase cDNA-mRNA Hybridization

The concentration of total cellular RNA from adrenal medullae was determined by extraction using a modification of our previously published method (31). Sonicated tissue (75 μl homogenate) was extracted with "RNAzolB" (a mixture of phenol, guanidinium thiocyanate, Biotecx, Friendswood, TX) (7). The integrity of the isolated RNA was verified using agarose (1%) gel electrophoresis in comparison with 18S and 28S RNA standards (Sigma, St. Louis, MO). The pBR322 recombinant plasmid containing the TH.36 cDNA probe (5), kindly supplied by Dr. Karen O'Malley (Washington University, School of Medicine), was grown in *E. coli* and plasmid DNA was isolated by standard procedures. The cDNA was excised from the plasmid DNA with Eco RI and purified by electrophoresis on a 1% agarose gel. The cDNA was recovered from the agarose using the "geneclean II" kit (Bio 101 Inc., La Jolla, CA). Briefly, the cDNA band was exised and dissolved in 3 vol. of 6 m NaI at 50°C. A "glassmilk" suspension was added and allowed to incubate on ice for 5 min. The glassmilk/cDNA complex was centrifuged and the resulting pellet was washed several times with "new wash" solution (NaCl/ethanol/water mix, pH 7–8.5 with Tris). The glassmilk/cDNA complex was repelleted and an equal volume of the buffer (1 mM EDTA, 10 mM Tris) was added to resuspend the pellet. The suspension was then incubated at 50°C for 3 min, the glassmilk was repelleted, and the resultant supernatant containing the cDNA was removed.

Several concentrations of serially diluted RNA samples were immobilized on nylon membranes (Gene Screen, New England Nuclear, Boston, MA) using a Bio-Rad slot blot apparatus. The filters were baked at 80°C for 2–4 h, then prehybridized using 25 mM potassium phosphate, 5 × SSC, 5 × Denhardt's solution, 50 μg/ml denatured salmon testes DNA, and 50% formamide. After incubation for 14–16 h at 42°C, filters were hybridized with a ^{32}P random primer-generated rat TH.36 cDNA probe. After hybridization for 14–16 h at 42°C, the filters were washed and exposed to x-ray film (Kodak X-AR, Rochester, NY) for 72 h. The developed autoradiograms were scanned using a Bio-Rad Model 620 Video Densitometer. The optical density per μg of total cellular RNA was calculated by comparison with internal laboratory standards of rat adrenal medullary RNA present on each nylon membrane. Experimental values were within the linear range of the standards.

Northern analysis indicated this cDNA probe hybridized to a single mRNA species in rat adrenal medulla at 2.1 kb (data not shown), which is similar to published reports (13,18).

TH Protein Levels

TH protein levels were determined with modification of our previously described methods (26). Tissue homogenates were diluted in phosphate buffer containing 1% SDS and boiled for 10 min. Samples were then dot-blotted onto nitrocellulose membranes (Bio-Rad, Richmond, CA) using a constant volume of 1 μl/dot and four concentrations of protein up to 1 μg/μl. Nitrocellulose blots were then incubated with 2% gelatin in phosphate-buffered (pH 7.5) saline containing 0.1% Tween-20 (PBS-T) at room temperature for 1 h. The blots were washed several times with PBS-T and incubated with polyclonal antibody to TH IgG (Pel-Freez Biologicals, Rogers, AR) in fresh PBS-T at room temperature for 1 h. Blots were washed and incubated with HRP-labeled donkey anti-rabbit IgG (Amersham Life Sciences, Arlington Heights, IL) at room temperature for 1 h. The blots were then washed and incubated with ECL detection reagents 1 and 2 (Amersham Life Sciences) at room temperature for 1 min. The blots were allowed to air dry for 10 min and were then exposed from 15 s to 5 min on X-Omat AR film (Eastman Kodak, Rochester, NY). The resulting autoradiographs were quantitated with a Bio-Rad Model 620 video densitometer (Bio-Rad).

TABLE 1

EFFECTS OF COLD EXPOSURE AND AGE ON BODY AND ADRENAL PARAMETERS IN F-344 RATS

	3 Months		24 Months	
	26°C	48 h (8°C)	26°C	48 h (8°C)
Body weight (g)	318 ± 9	293 ± 4	444 ± 10*	416 ± 12
Adrenal medullae wt (mg)	25 ± 1	38 ± 2†	40 ± 4*	39 ± 3
Adrenal medullary protein (mg)	3.4 ± 0.1	4.4 ± 0.2†	3.5 ± 0.3	3.8 ± 0.2

Values represent the means ± SE of 8–10 rats in each young and old group. Adrenal medullae weight represent a pair of medullae from the same animal ($p < 0.05$ by Student's t-test).

*$p < 0.001$ for difference with age.

†$p < 0.01$ for difference from age-matched controls following cold exposure.

Statistical Analysis

Means and SEMs were calculated from values obtained from a pair of adrenal medullae from individual animals of each age and treatment group. Comparison of means among different age and treatment groups were made by Student's t-test. Differences were considered significant when $p < 0.05$.

RESULTS

Body temperature was assessed in control rats maintained at 26°C and cold-exposed rats maintained at 8°C for 48 h (25). After a brief period of adjustment, body temperature was indistinguishable between cold-exposed and control rats of both ages. The body temperature of the rats in all the groups became synchronized and displayed the normal circadian rhythm with elevated nighttime and reduced daytime temperatures. These data were published previously (25). All animals survived this mild cold exposure with no ill effects.

Body weights and adrenal weights were greater in old compared with young rats (Table 1). Following a 48-h cold exposure at 8°C, body weights decreased in young rats, although it was not significant (Table 1). As expected, adrenal medullae weights were greater in old animals compared with young animals. Cold exposure caused a significant increase in adrenal medulla weights in young rats but not in old rats (Table 1). The total amount of protein recovered from homogenates was greater with age and in the cold-exposed animals of either age (Table 1). In light of these increases in adrenal protein with age and cold exposure, TH activity, immunoreactivity, and mRNA were normalized per adrenal medullae.

To determine the effect of cold and age on the capacity for CA biosynthesis, TH activity was assessed in homogenates of adrenal medullae following a 48-h cold exposure in young and old rats. Among control groups (26°C), TH activity was significantly greater in the 24-month-old compared with the 3-month-old animals (Fig. 1). The consequences of cold exposure on TH activity were different in young and senescent rats. Among the 3-month-old animals, TH activity was significantly higher in the cold-exposed group compared with the young room temperature group. However, in the older rats, there was no significant difference in TH activity associated with cold exposure (Fig. 1).

To determine if the changes in TH activity with age and cold are results of alterations in the amount of TH protein, we assessed TH immunoreactivity. In young rats, TH immunoreactivity increased significantly following cold exposure at 8°C (Fig. 2). Although TH protein was elevated significantly with age, there was no change in TH immunoreactivity in 24-month-old animals after a 48-h cold exposure (Fig. 2).

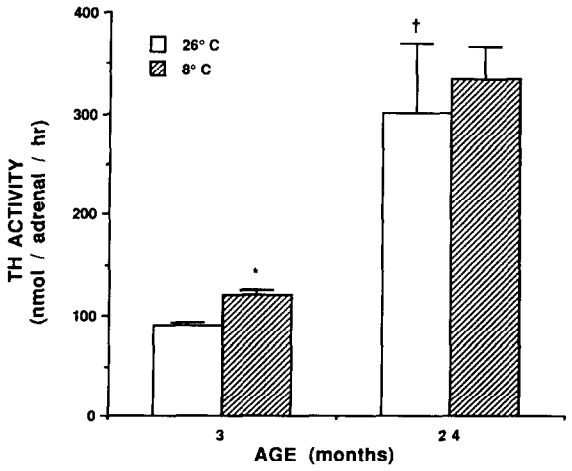

FIG. 1. TH activity with age and following 48-h cold exposure. Values represents mean ± SEM of 8–10 rats assayed in duplicate. *Significantly different from aged-matched control, $p < 0.005$. †Significantly different with age, $p < 0.001$.

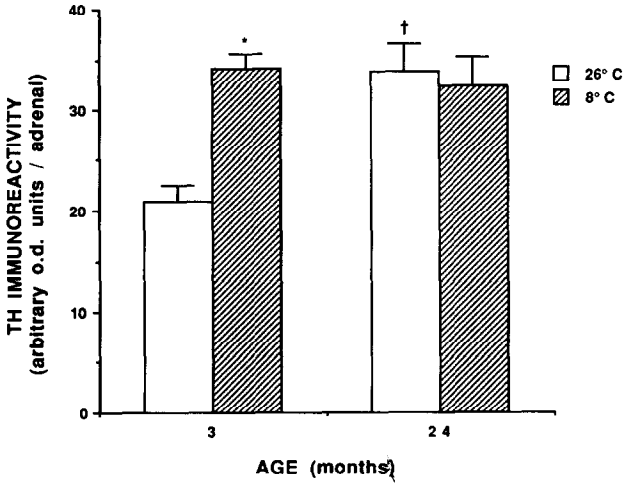

FIG. 2. Effects of age and 48-h cold exposure on TH immunoreactivity. Values represent mean ± SEM of 8–10 rats. *Significantly different from age-matched control, $p < 0.005$. †Significantly different from 3-month-old control, $p < 0.001$.

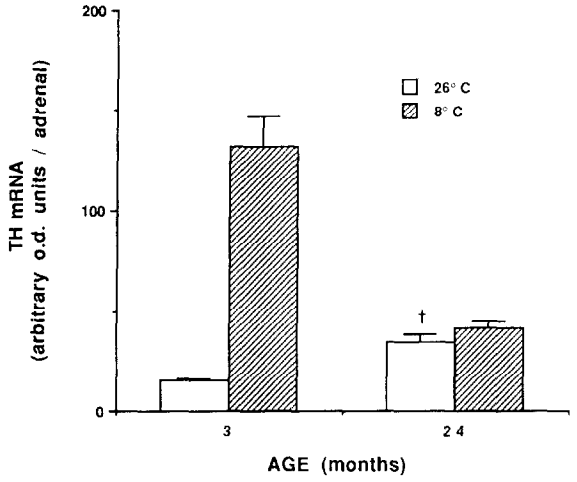

FIG. 3. TH mRNA with age and 48-h cold exposure. Values represent mean ± SEM of 8–10 rats. *Significantly different from age-matched control, $p < 0.005$. †Significantly different from 3-month-old controls, $p < 0.001$.

To assess whether alterations in TH activity and TH protein are a consequence of changes in gene expression, we measured TH mRNA levels by slot blot analysis, as shown in Fig. 3. Among control animals, a comparison between age groups revealed that TH mRNA was significantly ($p < 0.01$) elevated by 50% in adrenal medullae from old rats compared with young rats. This was similar to the increase in TH immunoreactivity and TH activity with age. In the young animals, TH mRNA in adrenals significantly increased with cold exposure. In contrast to the young rats, there was no significant effect of cold exposure on TH mRNA levels in adrenals in 24-month-old animals (Fig. 3).

The effects of cold exposure on TH activity, TH immunoreactivity, and TH mRNA were also examined as changes per unit protein and RNA. Even though cold exposure resulted in a generalized increase in protein per adrenal medulla, a specific increase in TH protein as measured by TH immunoreactivity was observed over and above the increase in total protein in young rats (Table 2). This augmentation in TH protein did not occur in the senescent rats. Similarly, there was a specific increase in TH mRNA per unit total RNA in the young rats following cold exposure but not in the older rats (Table 2). In contrast, TH activity per mg protein did not increase with cold exposure in rats of either age (Table 2).

DISCUSSION

Chronic cold exposure is associated with a gradual increase in the activity of TH, the key enzyme in CA biosynthesis. In addition, with cold stimulus, the relative abundance and the cell-free translation activity of TH mRNA increases in the sympathoadrenal system (3,8,11,27,30).

One of the most serious consequences of the aging process is an inability to respond to environmental stimuli. Older rats have decreased CA in the heart and increased CA in the adrenal medulla (24), and these downregulated and upregulated systems, respectively, may no longer be capable of responding to cold stimulus. Older rats do not maintain their body temperature as well as younger rats when exposed to the cold. This may be due to impaired thermoregulatory mechanisms with age (25). Thus, in older rats, the stimulus for CA increase should be reinforced by a lack of negative feedback (i.e., body temperature remains low). In the face of this reinforced stimulus, we determined the response of TH activity in the older animals compared with younger animals following cold exposure.

As expected, following 48 h of cold exposure, the young rats demonstrated augmentations in TH activity, TH immunoreactivity, and TH mRNA. Some of these increases were due to a combination of both elevated glandular protein and increased activity per unit of protein following cold exposure. In contrast, in the senescent rats specific levels per unit protein or per pair of adrenals were unchanged following cold stimulus. This suggests that induction of TH activity is impaired in senescent rats following cold exposure. Alternatively, the maximum capacity for synthesis of TH in the old rats has been reached and they simply cannot respond to additional stimulation. However, our previous data from exercise-trained rats suggest this is not the case. In contrast to cold stimulus, which increases the synthesis and circulating levels of CAs, exercise training decreased synthesis as well as plasma CAs. Previously, we reported that TH activity and TH mRNA were reduced in young animals following training, but there were no effects of training in the old F-344 rats (31). Collectively, our data suggest that the ability to both turn on and turn off the synthesis of CAs is blunted with age. Further supporting this loss of plasticity with age is a study by Strong et al. (29) in which TH mRNA and TH activity in the adrenal medulla were assessed after reserpine treatment. TH mRNA increased after reserpine treatment in all ages; however, TH activity increased only in the 2-month-old animals. These results suggests impaired posttranscriptional or posttranslational regulation with age, whereas our results suggest a transcriptional or mRNA stability dysfunction with age.

There is some evidence that the impaired synthesis of TH

TABLE 2

EFFECTS OF COLD EXPOSURE AND AGE ON TH ACTIVITY, TH IMMUNOREACTIVITY, AND TH mRNA PER UNIT PROTEIN OR RNA

	3 Months		24 Months	
	26°C	48 h (8°C)	26°C	48 h (8°C)
TH activity (nmol/mg protein/h)	27 ± 0.3	28 ± 1.1	80 ± 12*	85 ± 7
TH immunoreactivity (OD units/μg protein)	64 ± 5	86 ± 2†	85 ± 4*	85 ± 7
TH mRNA (OD units/μg RNA)	14.1 ± 0.7	96.8 ± 5.8†	29.8 ± 2.1*	24.9 ± 2.1

Values represent the means ± SE of 8–10 per age and treatment group ($p < 0.05$ by Student's t-test).
*Significantly different with age ($p < 0.005$).
†Significantly different from age-matched control (26°C) ($p < 0.001$).

following cold exposure is a failure of the chromaffin cells rather than of neuronal origin. In the older rat, the sympathetic afferent nerves innervating the adrenal medulla have increased neural activity in the resting condition (15). Sympathetic outflow has not been assessed with age following cold exposure; however, plasma concentrations of epinephrine increase equally in young and old rats after acute cold exposure (1). This latter observation reflects the release of stored epinephrine and not necessarily new synthesis. Collectively, these data coupled with our observation of deficient epinephrine synthesis in senescent rats following cold exposure suggest that the adrenals are receiving adequate signal to respond to the cold stress and are releasing stored epinephrine but are not synthesizing new epinephrine. If neuronal stimulation of the adrenal gland is unchanged, then the lack of new epinephrine synthesis in the senescent rats is a failure of the chromaffin cells to response to the cold stimulation.

The primary finding from these studies is that chronic mild cold exposure is associated with an increase in TH gene expression, TH immunoreactivity, and TH activity in the adrenal medullae of young rats but not old rats. These data suggest that the induction of TH activity is impaired in senescent rats following cold exposure and that there is a loss of plasticity with respect to the TH gene expression during aging. Further investigations are needed to determine the underlying mechanisms governing the inability of the catecholaminergic system of older rats to respond and adapt to environmental stimuli.

ACKNOWLEDGEMENTS

This work was supported by grants from the Merit Review Program of the Department of Veterans Affairs Medical Research Service and from the American Heart Association, Florida Affiliate. We thank Dr. Karen O'Malley of Washington University School of Medicine, St. Louis, MO, for providing the TH.36 cDNA probe. We also thank Janice Dolson and Anne Crawford for manuscript preparation and Janet Wootten for editing the manuscript.

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