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Induction of tyrosine hydroxylase by forskolin: modulation with age

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Abstract

With aging, circulating catecholamines are elevated in both humans and animals. This may be related to the increased basal levels of tyrosine hydroxylase messenger RNA (mRNA) levels and tyrosine hydroxylase enzyme activity in the adrenal medulla of senescent compared with younger animals. In addition, tyrosine hydroxylase gene expression in the senescent rat is resistant to further stimulation by cold exposure as compared with younger animals. Collectively, these observations suggest either that tyrosine hydroxylase expression is already maximally stimulated in senescent rats or that tyrosine hydroxylase gene induction pathways are impaired with senescence. To help distinguish between these possibilities, we examined the induction of tyrosine hydroxylase mRNA, tyrosine hydroxylase immunoreactivity and tyrosine hydroxylase enzyme activity in the adrenal medulla following forskolin administration to young and old F-344 rats. Forskolin at doses of 1.8 and 3.5 mg/kg increased tyrosine hydroxylase mRNA levels 2.5-fold in adrenal medulla from young rats but did not increase either tyrosine hydroxylase immunoreactivity or tyrosine hydroxylase enzyme activity 5 h after administration. Prolonged treatment with forskolin (3 doses, 12 h apart) increased tyrosine hydroxylase mRNA levels and tyrosine hydroxylase immunoreactivity and tyrosine hydroxylase enzyme activity. In senescent rats, the baseline level of tyrosine hydroxylase mRNA was more than 2-fold higher compared with young rats. A single injection of the lower dose of forskolin increased tyrosine hydroxylase mRNA levels by the same increment in senescent as compared with young rats. These data indicate that the tyrosine hydroxylase gene in the adrenal medulla from senescent rats is still capable of further stimulation.

Keywords: Adrenal medulla; Catecholamine synthesis; (F-344 rat)

1. Introduction

Circulating catecholamines are elevated in both humans and laboratory animals with aging (Avakian et al., 1984; Chiueh et al., 1980; Cizza et al., 1995; Esler et al., 1981; Hoeldtke and Cilmi, 1985; Ito et al., 1986; Ziegler et al., 1976). These elevations in circulating catecholamines may be related to the increased release of catecholamines with age from sympathetic ganglia and adrenals (Ito et al., 1986; Roberts and Tümer, 1987; Banerji et al., 1984), which, in turn, may be the result of the progressive increase in the synthesis of both epinephrine and norepinephrine with age (Roberts and Tümer, 1987). Tyrosine hydroxylase is the rate limiting enzyme in the synthesis of catecholamines (Nagatsu et al., 1964). We, as well as others, have reported that basal levels of tyrosine hydroxylase messenger RNA (mRNA) and tyrosine hydroxylase enzyme activity are 2–3-fold higher in senescent compared with younger animals (Tümer and LaRochelle, 1995; Kedzierski and Porter, 1990; Tümer et al., 1992; Voogt et al., 1990).

Cold exposure is known to elevate tyrosine hydroxylase mRNA levels, as well as the synthesis and release of catecholamines in the peripheral and the central nervous system, including the brain (Zigmond et al., 1974), the adrenal medulla (Tümer and LaRochelle, 1995; Baruchin et al., 1990; Fluharty et al., 1985a,b; Kvetnansky et al., 1971; Stachowiak et al., 1985, 1986) and the heart (Fluharty et al., 1985a). Moreover, we have previously demonstrated that chronic cold exposure is associated with an increase in tyrosine hydroxylase gene expression, tyrosine hydroxylase immunoreactivity, and tyrosine hydroxylase activity in the adrenal medullae of young rats but not old rats (Tümer and LaRochelle, 1995). These data suggest that the induc-

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tion of tyrosine hydroxylase gene expression and the subsequent increase in tyrosine hydroxylase activity are impaired in senescent rats following cold exposure.

The promoter region of the gene for tyrosine hydroxylase has numerous regulatory elements including the cAMP response element (Yoon and Chikaraishi, 1992; Kim et al., 1993). This element is essential for both the basal level of tyrosine hydroxylase transcription as well as the cyclic AMP (cAMP)-mediated induction (Kim et al., 1993). The exact physiologic role of cAMP in regulating the expression of tyrosine hydroxylase is unknown, but cAMP may be involved in either the stress-induced or cold-induced increases in tyrosine hydroxylase expression and the subsequent synthesis of catecholamines in the adrenal medulla.

Investigations of cAMP-mediated increases in tyrosine hydroxylase gene expression and tyrosine hydroxylase enzyme activity have been mostly limited to in vitro culture systems (Hwang et al., 1994; Wessels-Reiker et al., 1991; Kedzierski et al., 1994; Aguila-Mansilla et al., 1993). cAMP or agents that increase cAMP have been shown to elevate tyrosine hydroxylase mRNA in bovine chromaffin cells (Hwang et al., 1994), PC 12 cells (Wessels-Reiker et al., 1991), hypothalamic cells (Kedzierski et al., 1994) and hypothalamic slices (Aguila-Mansilla et al., 1993). One in vivo investigation using rats demonstrated that following injection of forskolin directly into the substantia nigra, tyrosine hydroxylase mRNA and tyrosine hydroxylase enzyme activity increased (Leviel et al., 1991).

The present study examines the cAMP-mediated tyrosine hydroxylase gene expression in vivo by administration of forskolin, a direct activator of the enzyme adenylyl cyclase. Furthermore, the observation that tyrosine hydroxylase gene expression in the senescent rat is resistance to further physiological stimulation coupled with the observation that tyrosine hydroxylase gene expression is elevated with senescence suggests either that tyrosine hydroxylase expression is already maximally stimulated in senescent rats or that tyrosine hydroxylase gene induction pathways are impaired with senescence. To help distinguish between these possibilities, we examined the induction of tyrosine hydroxylase mRNA, tyrosine hydroxylase immunoreactivity and tyrosine hydroxylase enzyme activity in the adrenal medulla following injection of forskolin to young and old F-344 rats.

2. Materials and methods

2.1. Animals and chemicals

Male F-344 NNia rats of 4–6 (young) and 24 (senescent) months of age were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA) under contract with the National Institute on Aging. Upon 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 of both ages were housed individually in micro-isolated cages and maintained on Purina Rat Chow ad libitum with a 12/12 h light-dark cycle (06:00–18:00 h). Experiments were begun 60–90 min after the beginning of the light cycle. Forskolin (Calbiochem, La Jolla, CA, USA) was dissolved in 50% dimethyl sulfoxide, 25% ethanol and 25% H_2O.

2.2. Tissue preparation

Animals were anesthetized with pentobarbital, and the adrenal glands were removed quickly and immediately frozen by immersion in liquid nitrogen. Tissues were stored at −80°C. At the time of the assay, while the tissue was in a partially frozen state, the adrenal glands were decapsulated and the medullae were separated from the cortex. Adrenal medullary 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 (1976).

2.3. Tyrosine hydroxylase activity

Tyrosine hydroxylase activity was measured using a radioenzymatic assay as described previously (Tümer and LaRochelle, 1995) and based on a modification of the assay by Reinhard et al. (1986). Briefly, 25 μl of homogenate were analyzed at pH 7.0 in the presence of 6-methyl-5,6,7,8-tetrahydropterin hydrochloride (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 absorption of the isotopic substrate (and its metabolites) by an aqueous slurry of activated charcoal. Unbound ^3H_2O was analyzed by liquid scintillation spectrometry.

2.4. Tyrosine hydroxylase mRNA

Tyrosine hydroxylase mRNA was determined in the adrenal medulla using our previously published method (Tümer and LaRochelle, 1995). Briefly, sonicated tissue (75 μl homogenate) was extracted with RNAzolB (a mixture of phenol and guanidinium thiocyanate, Biotecx, Friendswood, TX, USA) (Chomczynski and Sacchi, 1987). 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, USA). The pBR322 recombinant plasmid containing the tyrosine hydroxylase.36cDNA probe, kindly supplied by Dr. Karen O'Malley (Washington University, School of Medicine), was grown in *Escherichia coli*, and plasmid DNA was isolated by standard procedures (Tümer and LaRochelle, 1995). Several concentrations of serially diluted RNA samples were immobilized on nylon membranes (Gene Screen, New England Nuclear, Boston, MA, USA) using a Bio-Rad slot blot apparatus. After prehybridization, filters

were hybridized with a ^{32}P random primer-generated rat tyrosine hydroxylase.36cDNA probe. After hybridization, the filters were washed and exposed to phospho screen for 72 h using PhosphoImager (Molecular Dynamics, Sunnyville, CA, USA). The screens were scanned, and volumes for each sample were calculated from the counts per pixel using Image Quant software (Molecular Dynamics). The images (volume) were normalized 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.

2.5. Tyrosine hydroxylase immunoreactivity

Tyrosine hydroxylase protein levels were determined using our previously described methods (Tümer and LaRochelle, 1995). Briefly, tissue homogenates were diluted in phosphate buffer containing 1% sodium lauryl sulfate and boiled for 10 min. Samples were then dot-blotted onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). Immunoreactive protein was assessed by using polyclonal antibody to tyrosine hydroxylase (Pel-Freez Biologicals, Rogers, AR, USA) and horseradish peroxidase-labeled donkey anti-rabbit serum (Amersham Life Sciences, Arlington Heights, IL, USA) and visualized by using chemiluminescent detection (Amersham Life Sciences), quantified by video densitometry (Bio-Rad). This antibody recognizes a single 60 kDa band on Western blots.

3. Results

In previous studies, we determined that young rats could tolerate a dose of forskolin of 3.5 mg/kg without any noticeable distress. However, the highest tolerable dose in senescent rats was 1.8 mg/kg (Scarpace and Matheny, 1996). Forskolin is a potent stimulant of many physiological functions. We previously demonstrated that the two doses of forskolin employed in this study stimulated a sustained 30% increase in oxygen consumption for 60 min (Scarpace and Matheny, 1996). To determine whether the expression of tyrosine hydroxylase increases following forskolin administration, tyrosine hydroxylase activity, tyrosine hydroxylase immunoreactivity, and tyrosine hydroxylase mRNA levels in the adrenal medulla were examined 5 h after forskolin administration in young rats. Both the low and high doses of forskolin increased tyrosine hydroxylase mRNA levels by greater than 2.5-fold (Table 1). The efficacy of both doses of forskolin to increase tyrosine hydroxylase mRNA levels was equivalent. Not surprisingly, neither dose of forskolin increased tyrosine hydroxylase immunoreactivity nor tyrosine hydroxylase enzyme activity (Table 1). Other studies have indicated that tyrosine hydroxylase protein and tyrosine hydroxylase activity do not increase until 12–48 h after cold stimulation (Tank et al., 1985; Baruchin et al., 1990). Forskolin administration had no effect on the adrenal medullary weight, total protein content or extractable RNA (data not shown).

To determine whether forskolin administration also stimulates tyrosine hydroxylase gene expression in senescent rats, the lower dose of forskolin (1.8 mg/kg) was administered to senescent rats followed by killing 5 h later. The body weights of the older rats were slightly greater than those of the 6-month-old rats (436 ± 9 vs. 377 ± 4, $P < 0.001$), as were the weights of the adrenal medulla (41.9 ± 10.2 vs. 30.6 ± 4.3 mg, $P < 0.002$). Similar to our previous findings (Tümer and LaRochelle, 1995), tyrosine hydroxylase mRNA levels in control senescent rats were greater than 2-fold higher than the mRNA levels in control young rats (Fig. 1). Following forskolin administration, the incremental increase in tyrosine hydroxylase mRNA was the same in both young and senescent rats (71 vs. 71, Fig. 1); however, because of the higher baseline value in the older rats, the percent increase in these rats was less (79% vs. 178%). After forskolin stimulation, the levels of tyrosine hydroxylase mRNA remained significantly higher in the adrenal medulla from senescent compared with young rats (Fig. 1). In control rats, as reported previously (Tümer and LaRochelle, 1995) the level of tyrosine hydroxylase activity was substantially greater in the adrenal medulla from the older compared to the young rats (58.0 ± 4.3 nmol/mg per h vs. 34.1 ± 1.3, $P < 0.001$). Similar to the findings in younger rats, tyrosine hydroxylase enzyme activity did not increase at 5 h following forskolin admin-

Table 1
Effect of forskolin on tyrosine hydroxylase activity, immunoreactivity, and mRNA levels

Treatment	Tyrosine hydroxylase mRNA (OD units/μg RNA)	Tyrosine hydroxylase immunoreactivity (OD units/μg protein)	Tyrosine hydroxylase activity (nmol/mg per h)
Control	100 ± 9	100 ± 11	34.1 ± 1.3
Forskolin (1.8 mg/kg)	275 ± 24 [a]	88.5 ± 7.6	36.3 ± 1.8
Forskolin (3.5 mg/kg)	259 ± 39 [a]	80.9 ± 9.9	29.6 ± 1.6

All animals were sacrificed 5 h after forskolin administration. Data represents the mean ± S.E. of 11 control, six low-dose forskolin, and eight high-dose forskolin administered rats. Values for control tyrosine hydroxylase mRNA and tyrosine hydroxylase immunoreactivity were arbitrarily set to 100.
[a] $P < 0.001$ for difference with treatment by one-way ANOVA; $P < 0.01$ for difference from control values by Dunnett multiple comparison test.

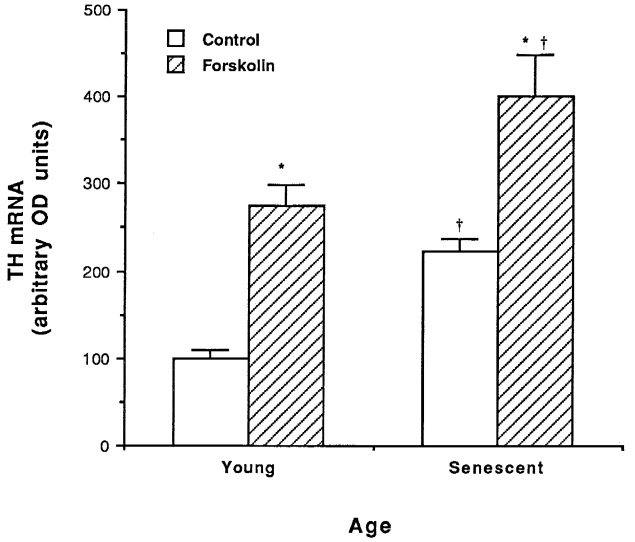

Fig. 1. Tyrosine hydroxylase mRNA levels in the adrenal medulla of young and senescent rats administered a single dose of forskolin (1.8 mg/kg). Data represent the mean ± SE of 4–6 rats in each group. The tyrosine hydroxylase mRNA level for young controls was arbitrarily set to 100. * $P < 0.001$ for difference with forskolin treatment by two-way ANOVA; $P < 0.001$ (young) and $P < 0.003$ (senescent) for difference with forskolin from corresponding controls by Scheffe's post-hoc analysis. † $P < 0.001$ for difference with age by two-way ANOVA. $P < 0.001$ (control) and $P < 0.05$ (forskolin) for difference with age by Fisher's protected LSD post-hoc analysis.

istration in the senescent rats (58.0 ± 4.3 nmol/mg per h control vs. 57.1 ± 3.5 forskolin).

The lack of an increase in tyrosine hydroxylase activity in the face of increasing tyrosine hydroxylase mRNA levels suggests that the interval between forskolin administration and killing is insufficient for translation or post-translation processing of tyrosine hydroxylase message. To test this, three doses of forskolin were administered 12 h apart to young rats followed by killing 5 h after the last dose. In these prolonged forskolin-treated rats, tyrosine hydroxylase mRNA levels in the adrenal medulla increased 2.6-fold (Table 2). This was similar to the increase following a single injection of forskolin (Table 1). However, in contrast to the rats administered a single forskolin injection, both tyrosine hydroxylase immunoreactivity and tyrosine hydroxylase enzyme activity also increased in the rats administered multiple forskolin injections (Table 2).

4. Discussion

The adrenal medullae from senescent rats are resistant to the cold-induced elevation of tyrosine hydroxylase mRNA and the subsequent increase in tyrosine hydroxylase immunoreactivity and tyrosine hydroxylase enzyme activity (Tümer and LaRochelle, 1995). These observations suggest that the tyrosine hydroxylase induction pathway is impaired with senescence. The exact mediator of the cold-induced stimulation of tyrosine hydroxylase gene expression is unknown, and this increased expression is most likely the consequence of the integration of several signals, including both the cAMP and AP-1 signal transduction pathways (Kim et al., 1993; Miner et al., 1992). Impairment of one or more of these signals with age could result in a failure in inducible tyrosine hydroxylase gene expression. Alternatively, because basal tyrosine hydroxylase mRNA levels, tyrosine hydroxylase immunoreactivity, and tyrosine hydroxylase enzyme activity are 2- to 3-fold higher in older compared with younger rats (Tümer and LaRochelle, 1995), tyrosine hydroxylase gene expression could already be maximally activated in the basal state of the senescent rats, such that further stimulation by cold exposure is ineffectual. The present study examined the latter possibility by administering forskolin to young and senescent rats.

Forskolin is a direct activator of the enzyme adenylyl cyclase and results in a rise in intracellular cAMP levels (Seamon and Daly, 1986). cAMP can both activate existing tyrosine hydroxylase enzyme by promoting phosphorylation of inactive enzyme and can stimulate the syntheses of new enzyme through activation of the cAMP response element on the promoter region of the tyrosine hydroxylase gene (Masserano and Weiner, 1979). In the present study, forskolin increased tyrosine hydroxylase mRNA levels 5 h after administration of a single dose of forskolin. In young rats, both a low and high dose of forskolin resulted in the same elevation of tyrosine hydroxylase mRNA levels. As expected, neither tyrosine hydroxylase protein nor tyrosine hydroxylase activity increased at 5 h following forskolin administration. Five hours may not have been sufficient time for post-transcriptional processing of the new tyrosine hydroxylase message. However, despite the insufficient time interval for the synthesis of

Table 2
Effect of multiple doses of forskolin administration on tyrosine hydroxylase activity and tyrosine hydroxylase mRNA levels

Treatment	Tyrosine hydroxylase mRNA (OD units/μg RNA)	Tyrosine hydroxylase immunoreactivity (OD units/μg protein)	Tyrosine hydroxylase activity (nmol/mg per h)
Control	100 ± 16	100 ± 12	32.1 ± 2.9
Forskolin	268 ± 2.3 [a]	153 ± 17 [a]	41.6 ± 2.1 [a]

Forskolin (1.8 mg/kg) was administered 3 times, 12 h apart and the rats were sacrificed 5 h after the last dose. Data represent the mean ± S.E. of six rats in each group. Value for control mRNA was arbitrarily set to 100. [a] $P < 0.05$ (tyrosine hydroxylase activity and tyrosine hydroxylase immunoreactivity) and $P < 0.001$ (tyrosine hydroxylase mRNA) for difference from control values by one-way ANOVA.

new enzyme, one might expect that forskolin administration would increase tyrosine hydroxylase activity independent of new enzyme synthesis by promoting phosphorylation and activation of existing enzyme (Onali and Olianas, 1992). However, the tyrosine hydroxylase enzyme assay employed in the present study measures total enzyme activity and does not distinguish between phosphorylated and unphosphorylated enzyme.

When three doses of forskolin were administered over a 24-h period and the rats killed 5 h after the last dose, a different picture emerged. In this case, tyrosine hydroxylase mRNA levels remained elevated but with a concomitant increase in both tyrosine hydroxylase immunoreactivity and tyrosine hydroxylase activity. These data indicate that prolonged stimulation with forskolin can elevate tyrosine hydroxylase mRNA levels that subsequently result in new enzyme synthesis, similar to that observed following physiological stimulation.

The above results were in young animals. When the lower dose of forskolin was administered to senescent rats, tyrosine hydroxylase mRNA levels increased to the same extent as in younger rats. These data indicate that the already elevated level of tyrosine hydroxylase gene expression in the adrenal medulla from senescent rats is still capable of further stimulation. Whether these increased mRNA levels are translated into new protein in the senescent rats is unknown because longer time intervals after forskolin administration were not examined. However, with cold exposure, senescent rats do not demonstrate the increase in tyrosine hydroxylase mRNA that is observed in young rats. Collectively, these data suggest the inability of cold exposure to elevate tyrosine hydroxylase mRNA is not due to an already fully stimulated tyrosine hydroxylase gene in the senescent rats. These data coupled with our previous findings that cold exposure fails to increase tyrosine hydroxylase gene expression in senescent rats suggest that one or more of the cold-induced gene induction pathways is impaired with senescence.

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