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Induction of Tyrosine Hydroxylase and Neuropeptide Y by Carbachol: Modulation With Age

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With aging, circulating catecholamines are elevated in both humans and animals. This may be related to the increased basal levels of dopamine β-hydroxylase (DβH) and tyrosine hydroxylase (TH) mRNA levels and TH enzyme activity in the adrenal medulla of senescent compared with younger animals. Cold exposure induces TH and DβH mRNA, and the cholinergic pathway is believed to be involved in the cold-stimulated increase in TH expression in the adrenal medulla. However, TH gene expression in the senescent rat is resistant to stimulation by cold exposure, suggesting that the cholinergic pathway may be impaired with age in the adrenal medulla. To investigate this possibility, we administered carbachol (0.5 mg/kg ip, every 12 hours for 3 consecutive days), a mixed nicotinic-muscarinic agonist, to young (4-month-old) and senescent (24-month-old) male F-344 rats. We examined the induction of TH mRNA, TH immunoreactivity, and TH enzyme activity in the adrenal medulla in young and old rats. In addition DβH and NPY mRNA levels were determined in the adrenal medulla with or without carbachol administration. Basal levels of TH mRNA, TH immunoreactivity, and TH activity as well as DβH and neuropeptide Y (NPY) mRNA were 1.5- to 4-fold greater in the adrenal medullae of old rats compared with young rats. Carbachol administration increased TH mRNA, TH immunoreactivity, and TH activity as well as DβH and NPY mRNA to the same or a greater extent in the senescent compared with the young rats. The present study indicates that the cholinergic induction of TH or DβH are not impaired with age, and that senescent rats retain the capacity to respond to carbachol stimulation. The present findings cannot explain why the adrenal medullae from senescent rats are resistant to the cold-induced elevation of TH mRNA and TH activity observed in young rats.

ENVIRONMENTAL stress, such as cold exposure, is associated with increased plasma and adrenal medullary catecholamines. This is accompanied by an elevation in the catecholamine biosynthetic enzymes, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DβH), as well as increases in the mRNA species for these enzymes. In addition to catecholamines, neuropeptide Y (NPY) is also synthesized and colocalized with the epinephrine and norepinephrine within the adrenal medulla (1).

Circulating catecholamines are elevated in both humans and laboratory animals with aging (2–7). These elevations in circulating catecholamines may be related to the increased release of catecholamines with age from sympathetic ganglia and adrenals (6,8,9), which, in turn, may be the result of the progressive increase in the synthesis of both epinephrine and norepinephrine with age (8). TH is the rate-limiting enzyme in the synthesis of catecholamines (10). We, as well as others, have reported that basal levels of TH messenger RNA (mRNA) and TH enzyme activity are two- to threefold higher in senescent compared with younger animals (11–14). DβH is also an important enzyme in catecholamine biosynthesis, catalyzing the conversion of dopamine to norepinephrine, and the gene expression of DβH increases with age in the adrenal medulla (9). NPY, also synthesized in the adrenal medulla, is often regulated in parallel with catecholamine synthesis (1).

Cold exposure is known to elevate TH and DβH mRNA levels, as well as the synthesis and release of catecholamines in the peripheral and the central nervous system, including the brain (15), the adrenal medulla (11,16–21), and the heart (18). Moreover, we have previously demonstrated that chronic 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 (11). The cholinergic pathway is believed to be involved in the cold-stimulated increase in TH expression in the adrenal medulla. Both denervation and the ganglionic blocking agent, chlorisondamine, prevent the cold-induced increase in TH mRNA (21,22). Collectively, these data suggest that the impaired induction of TH gene expression following cold exposure in senescent rats may be the result of an impaired cholinergic pathway with age.

To investigate this possibility, we administered carbachol, a mixed nicotinic-muscarinic agonist to young (4-month-old) and senescent (24-month-old) male F-344 rats. We examined the induction of TH mRNA, TH immunoreactivity, and TH enzyme activity as well as DβH and NPY mRNA levels in the adrenal medulla, with or without carbachol administration, in young and old rats.

Methods

Animals and Experimental Design

Male F-344 NNia rats, 4 (young) and 24 (senescent) months of age, were obtained from Harlan Sprague-Dawley (Indianapolis, IN) 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 were housed individually and maintained on Purina Rat Chow ad libitum with a 12:12-hour light-dark cycle (06:00 to 18:00). Experiments were begun 60–90 minutes after the beginning of the light cycle. Experimental animals ($n = 6$) were injected with carbachol, 0.5 mg/kg ip, every 12 hours for 3 con-

secutive days. Control rats received an equivalent amount of saline. Animals were sacrificed 5 hours after the last dose following 80 mg pentobarbital administration.

Tissue Preparation

At sacrifice, 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, 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 (23).

TH Activity

TH activity was measured using a radioenzymatic assay as described previously (11) and based on a modification of the assay by Reinhard and colleagues (24). Briefly, 25 µL of homogenate were analyzed at pH 7.0 in the presence of cofactor (6-methyl-5,6,7,8-tetrahydropterin HCl, 1.5 mM) and [3,5-^{3}H]tyrosine (100 µM; 1 µCi/reaction), in a total volume of 50 µL for 15 minutes at 37°C. The assay is based upon the release of ^3H_2O from ^{3}H-[3,5]-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.

mRNA Levels

TH mRNA was determined in the adrenal medulla using our previously published method (11). Briefly, sonicated tissue (75 µL homogenate) was extracted with RNAzolB (a mixture of phenol and guanidinium thiocyanate, Biotecx, Friendswood, TX) (25). 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 TH.36cDNA probe was kindly supplied by Dr. Karen O'Malley (Washington University, School of Medicine, St. Louis), the DβH probe was kindly supplied by Dr. Esther Sabban (New York Medical College, NY), and the rat pre pro NPY cDNA was kindly provided by Dr. Janet Allen (University of Glasgow, UK). Several concentrations of serially diluted RNA samples were immobilized on nylon membranes (Gene Screen, New England Nuclear, Boston, MA) using a Bio-Rad (Richmond, CA) slot blot apparatus. After prehybridization, membranes were hybridized with ^{32}P random primer–generated probes. After hybridization, the membranes were washed and exposed to phospho screen for 72 hours using PhosphoImager (Molecular Dynamics, Sunnyville, CA). The screens were scanned, and volumes for each sample were calculated from the counts per pixel using Image Quant software (Molecular Dynamics). Nylon membranes were stripped and rehybridized to β-actin and glyceraldehyde-3-phosphate dehydrogenase. Images (volumes) 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.

TH Immunoreactivity

TH protein levels were determined using our previously described methods (11). Tissue homogenates were diluted in phosphate buffer containing 1% SDS and boiled for 10 minutes. Samples were then dot-blotted onto nitrocellulose membranes (Bio-Rad) 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 hour. 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 hour. Blots were washed and incubated with horseradish peroxidase–labeled donkey antirabbit IgG (Amersham Life Sciences, Arlington Heights, IL) at room temperature for 1 hour. The blots were then washed and incubated with chemiluminescent detection reagents 1 and 2 (Amersham Life Sciences, Arlington Heights, IL) at room temperature for 1 minute. The blots were allowed to air dry for 10 minutes and were then exposed from 15 seconds to 5 minutes on X-Omat AR film (Eastman Kodak, Rochester, NY). The resulting autoradiographs were quantitated with a Bio-Rad Model 620 video densitometer. This antibody recognizes a single 60-kDa band on Western blots.

Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA), and p values were reported when the main effect (carbachol, age, or interaction) was significant. When the main effect was significant, subgroups were examined by t test.

Results

Carbachol Administration to Young and Old Rats

As expected, the body weights of the older rats were significantly greater than those of the 4-month-old rats (424 ± 13 vs 300 ± 6 g, $p = .001$), as were the weights of the adrenal medulla (18.6 ± 1.2 vs 12.4 ± 1.0 mg, $p = .003$). Carbachol administration had no effect on body weight in either the young or old rats, but increased adrenal weight in young (15.5 ± 0.9 mg) and senescent (21.7 ± 1.1 mg) rats; only the increase in the young rats was significant ($p = .049$). The increase in weight of the adrenal medulla with carbachol administration was accompanied by a significant increase in protein in the young (1.44 ± 0.06 vs 1.77 ± 0.1 mg, $p = .02$) and a nonsignificant increase in the senescent (2.46 ± 0.11 vs 2.89 ± 0.18 mg, $p = .085$) rats.

TH mRNA Levels

Similar to our previous findings (11), TH mRNA levels in control senescent rats were greater than 1.5-fold higher than the mRNA levels in control young rats (Figure 1). Following carbachol administration, TH mRNA increased by 50% in adrenal medulla of young rats and by greater than twofold in old rats (Figure 1). In contrast, mRNA levels for β-actin and glyceraldehyde-3-phosphate dehydrogenase were decreased by 51.7 ± 5.2% and 58.5 ± 3.9% with age, respectively. There was no change in either β-actin or glyceraldehyde-3-phosphate dehydrogenase mRNA levels with carbachol treatment.

TH Activity

Among the control groups, TH activity per milligram protein was significantly elevated by greater than 1.5-fold in 24-month-old compared with 4-month-old control animals (Figure 2). Carbachol significantly elevated TH activity by 50% in adrenals

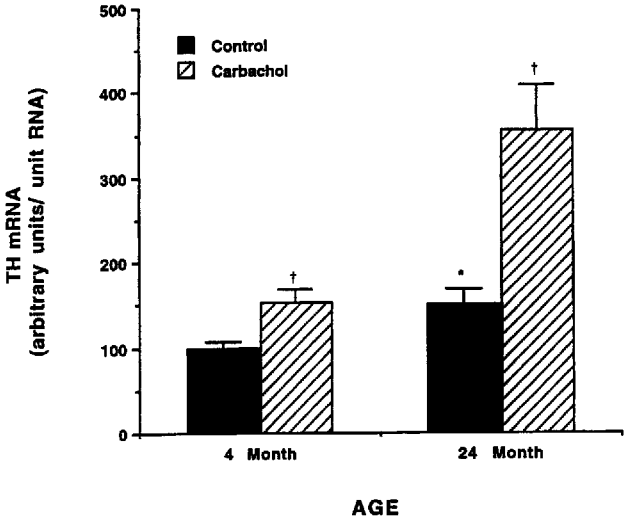

Figure 1. Carbachol (0.5 mg/kg every 12 hours for 3 consecutive days, ip) increased TH mRNA levels in the adrenal medulla 5 hours after sacrifice in both young and old rats. Data represent the mean ± *SE* of six rats. $p = .016$ for interaction between age and carbachol treatment. †$p = .0003$ for main effect of carbachol treatment by two-way ANOVA; $p = .015$ (young) and $p = .004$ (senescent) for difference with carbachol from corresponding controls by *t* test. *$p = .0003$ for main effect of age by two-way ANOVA. $p = .004$ (control) and $p = .023$ (carbachol) for difference with age by *t* test.

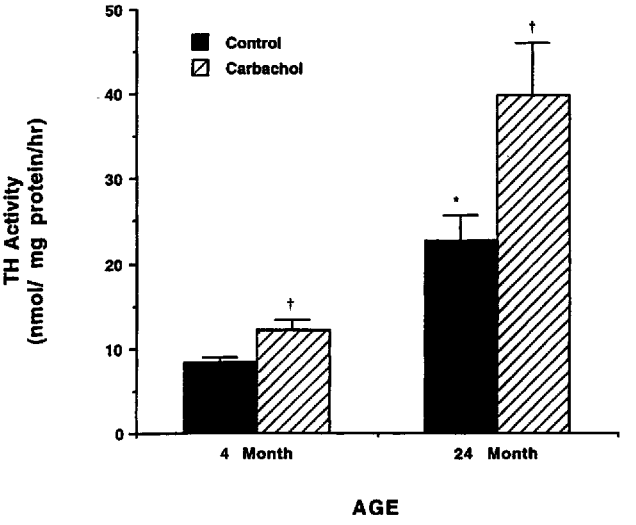

Figure 2. Carbachol elevated TH activity per milligram protein in adrenal medulla of both young and senescent animals. Data represent the mean ± *SE* of six rats. †$p = .018$ for main effect of carbachol treatment by two-way ANOVA; $p = .018$ (young) and $p = .041$ (senescent) for difference with carbachol from corresponding controls by *t* test. *$p = .0001$ for main effect of age by two-way ANOVA. $p = .001$ (control) and $p = .002$ (carbachol) for difference by *t* test. There was no significant interaction between age and carbachol.

of young and by 77% in the adrenals of old animals (Figure 2). Because both age and carbachol were associated with an increase in the protein content of the adrenal medulla, total TH activity per adrenal medulla was calculated. TH activity per adrenal was elevated by greater than fourfold in 24-month-old compared with 4-month-old control animals (12.0 ± 0.8 vs 55.8 ± 7.9 nmol/adrenal/h, $p = .0002$), and carbachol significantly elevated TH activity/adrenal in both young (21.1 ± 0.8 nmol/adrenal/h, $p = .0001$) and senescent (96.8 ± 7.9 nmol/adrenal/h, $p = .004$) rats.

TH Protein Levels

To determine if the effects of carbachol on TH activity were due to changes in the amount of TH protein, TH immunoreactivity was assessed in adrenals of young and old rats with and without carbachol administration. Among the control rats, TH protein level was significantly elevated by 65% in 24-month-old compared with 4-month-old animals (Figure 3). Carbachol significantly elevated TH protein by 50% in adrenal medulla of young rats and by 30% in old rats (Figure 3).

DβH and NPY mRNA Levels

Messenger RNA levels of DβH, an enzyme required for the synthesis of norepinephrine, were also assessed with age and following carbachol administration. Similar to other reports, DβH mRNA was significantly elevated by 33% in adrenals from old rats compared with young rats (Figure 4). Stimulation by carbachol, surprisingly, did not increase DβH mRNA in the adrenals of young rats ($p > .05$), but there was nearly a 60% increase in the senescent animals (Figure 4).

Previous studies have demonstrated that in addition to catecholamines, NPY is synthesized in the adrenal medulla and co-released with epinephrine and norepinephrine. Therefore, we

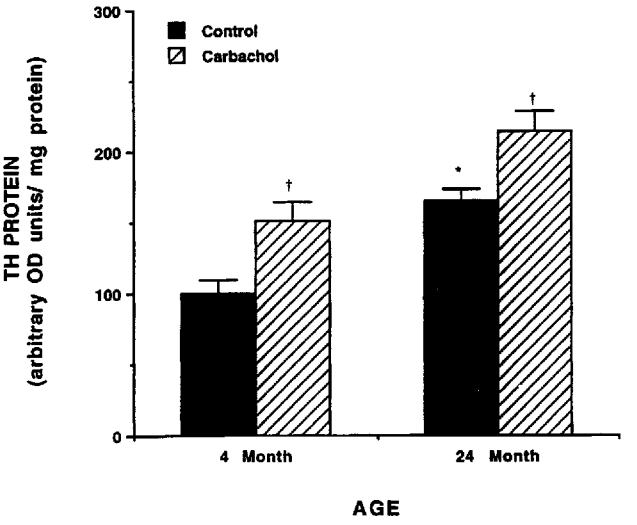

Figure 3. Carbachol stimulation of TH protein levels in adrenal medulla of young and senescent rats. Data represent the mean ± *SE* of six rats. †$p = .0005$ for main effect of carbachol treatment by two-way ANOVA; $p = .012$ (young) and $p = .016$ (senescent) for difference with carbachol from corresponding controls by *t* test. *$p = .0001$ for main effect of age by two-way ANOVA. $p = .0004$ (control) and $p = .02$ (carbachol) for difference with age by *t* test. There was no significant interaction between age and carbachol.

investigated the effects of age and carbachol administration on NPY mRNA in the adrenal medulla. Similar to TH mRNA, there was an age-related increase in NPY mRNA by nearly 50% (Figure 5). Carbachol increased NPY mRNA significantly in both adrenals from 4-month-old and 24-month-old animals, with an elevation of 72% in the young and greater than twofold in the old animals (Figure 5).

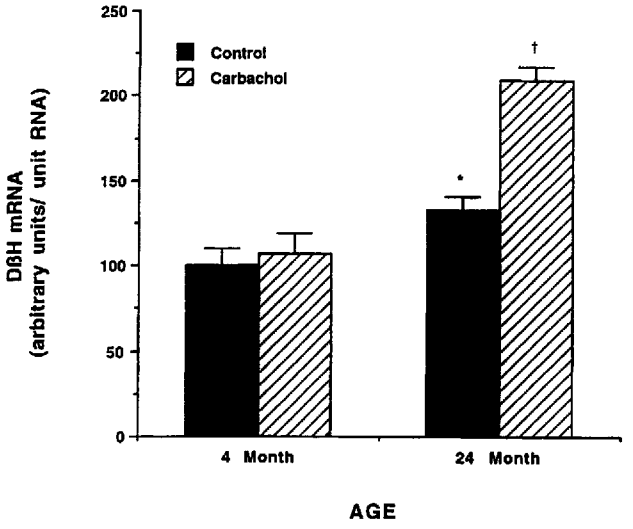

Figure 4. Effect of carbachol on DβH mRNA in the adrenal medulla of young and senescent animals. Data represent the mean ± *SE* of six rats. p = .002 for interaction between age and carbachol treatment. †p = .0004 for main effect of carbachol treatment by two-way ANOVA; p = .0001 (senescent) for difference with carbachol treatment from corresponding controls by t test. *p = .0001 for main effect of age by two-way ANOVA. p = .028 (control) and p = .0001 (carbachol) for difference with age by t test.

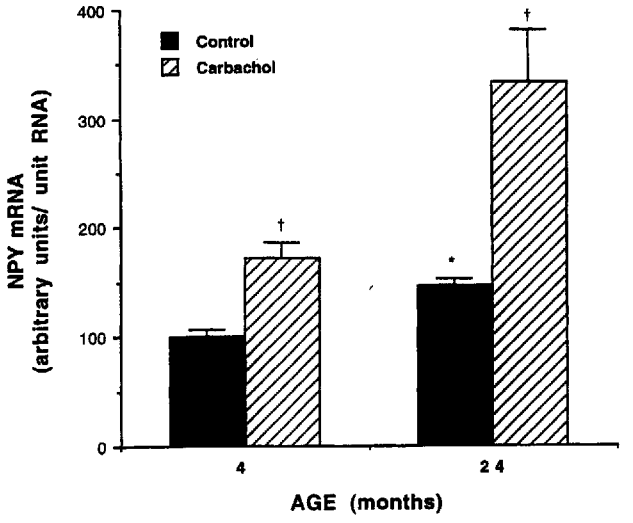

Figure 5. Carbachol increases NPY mRNA in adrenal medulla of young and senescent rats. Data represent the mean ± *SE* of six rats. †p = .0002 for main effect of carbachol treatment by two-way ANOVA; p = .002 (young) and p = .007 (senescent) for difference with carbachol from corresponding controls by t test. *p = .0018 for main effect of age by two-way ANOVA. p = .0008 (control) and p = .01 (carbachol) for difference with age by t test. There was no significant interaction between age and carbachol.

Discussion

Carbachol, a mixed nicotinic-muscarinic agonist, is a potent activator of TH and DβH gene expression. The mechanism by which this agonist activates gene transcription is complex and may involve activation of both the protein kinase A and protein kinase C signal transduction pathways (26). These signal transduction pathways activate at least two regulatory elements on the TH and DβH genes, AP-1 and the cAMP response element (CRE). The AP-1 regulatory element binds products of the immediate early genes, c-Fos– and c-Jun–related proteins, whereas CRE binds the cAMP response element–binding protein. Both the AP-1 and CRE regulatory elements are essential for the basal level of TH gene transcription (27,28). In addition, activation of the AP-1 regulatory element appears to be necessary for the enhanced expression following cold exposure (27,29). Similarly, the induction of both TH and DβH following immobilization stress is associated with increased AP-1 binding activity (30–32). The exact physiological role of the cAMP response element in regulating the expression of TH is unknown, but transcription factor binding to this element may be involved in either the stress-induced or cold-induced increases in TH expression and the subsequent synthesis of catecholamines in the adrenal medulla.

We previously demonstrated that chronic 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 (11). This lack of an increase in TH synthesis is apparently not a result of an inadequate signal to the adrenals (33). We previously demonstrated that choline acetyltransferase activity is unchanged in the adrenal medulla with age (13). Furthermore, in response to acute cold, plasma epinephrine increases equally in young and old rats (33). This increase in epinephrine represents release of stored adrenal medullary epinephrine and suggests that the adrenal gland is receiving adequate signal to respond to the cold stress. Collectively, these data suggest that the impairment in the cold-induced TH induction is within the adrenal gland; however, it is possible that an inadequate signal to the adrenal gland could account for the impaired cold response with age. We hypothesized that the failure of cold exposure to stimulate TH gene expression in the senescent rats may be a result of an impaired cholinergic pathway in the adrenal medulla with age. However, this was not the case. As reported previously, basal levels of TH mRNA, TH immunoreactivity, and TH activity were greater in the adrenal medullae of old rats compared with young rats. However, the present study indicates that carbachol administration increases TH mRNA, TH immunoreactivity, and TH activity to the same or a greater extent in the senescent compared with the young rats. Similarly, basal levels of DβH mRNA were elevated with age, and carbachol administration increased DβH mRNA levels to a greater extent in senescent compared with young rats. The present study indicates that the cholinergic induction of TH or DβH are not impaired with age, and that senescent rats retain the capacity to respond to carbachol stimulation.

NPY is synthesized in the adrenal medulla and is coreleased with epinephrine and norepinephrine (34). The physiological role of the secreted NPY is unclear; however, there is evidence of a role for NPY in the autocrine regulation of TH gene expression and activity (34) and as a vasoconstrictor (35). NPY is both a potent vasoconstrictor itself and can potentiate the norepinephrine-induced vasoconstriction (35), thus the adrenal medullary corelease of NPY with catecholamines may play a contributory role in the regulation of blood pressure (35). Physiological stimulators of TH gene expression such as immobilization stress or cold exposure also concomitantly increase NPY gene expression in the adrenal medulla (36). In the present study, we found that basal levels of NPY mRNA were

elevated with age in parallel with the elevated TH mRNA, similar to the effect on TH mRNA. A previous study indicated that NPY levels in the adrenal medulla were elevated in 16-month-old compared with 1.5-month-old Sprague-Dawley rats. The present study extends these finding to include NPY mRNA levels and 24-month-old rats, indicating that NPY mRNA levels increase into senescence. Carbachol administration increased NPY mRNA levels to a greater extent in senescent compared with young rats. These data suggest that, similar to the young animal, there is also a parallel regulation of both NPY and TH gene expression in senescent rats.

The present findings cannot explain why the TH induction pathway in the adrenal medullae from senescent rats is resistant to cold stimulation (11). The exact mediator of the cold-induced stimulation of TH 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 (28,30). Impairment of one or more of these signals with age could result in a failure in inducible TH gene expression. Alternatively, because basal TH mRNA levels, TH immunoreactivity, and TH enzyme activity are two- to threefold higher in older compared with younger rats (11), TH gene expression could already be maximally activated in the basal state of senescent rats, such that further stimulation by cold exposure is ineffectual. We previously reported that cold exposure increased transcription factor binding to the AP-1 regulatory element in the adrenal medulla and that the increase was equal in both young and senescent rats (37). Moreover, we found that administration of forskolin, a direct activator of adenylyl cyclase, stimulated TH gene expression in both young and old rats (38). These data suggest that individually both the adenylyl cyclase–cAMP response element pathway and the AP-1 response element pathway are unchanged with age. In addition, the present finding indicates that when both pathways are stimulated by carbachol, the responses are unchanged with age. Collectively, these data suggest that the inability of cold exposure to elevate TH mRNA is not due to an already fully stimulated TH gene in senescent rats, and that a third regulatory pathway must be involved in the impaired cold induction of TH gene expression in senescent rats.

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