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Expressions of cardiac sympathetic norepinephrine transporter and β_1-adrenergic receptor decreased in aged rats*

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Abstract: Evidence suggests that the deterioration of communication between the sympathetic nervous system and cardiovascular system always accompanies the aging of human and animals. Cardiac sympathetic norepinephrine (NE) transporter (NET) on presynaptic membrane is a predominant component to eliminate released NE in the synaptic cleft and maintains the sensitivity of the β-adrenergic receptor (β-AR). In the present study, we investigated NET and β_1-AR mRNA levels and sympathetic nerve density in cardiac sympathetic ganglion and left ventricular myocardium in 2- and 16-month-old rats with Northern blot analysis and immunohistochemistry. The expression levels of NET mRNA, NET protein and β_1-AR mRNA in the ganglia or myocardia of 16-month-old rats were markedly reduced by 67%, 26%, and 43%, respectively, in comparison with those in 2-month-old rats. Our results also show that aging induces a strong decrease of the catecholaminergic nerve fiber density.

Key words: Norepinephrine transporter (NET), β_1-adrenergic receptor (β_1-AR), Cardiac sympathetic ganglion, Aging
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INTRODUCTION

There are several lines of evidence suggesting that advanced aging in human and animals is accompanied by deterioration of communication between the sympathetic nervous system and cardiovascular system (Lakatta, 1993; Esler *et al*., 1995; Seals and Esler, 2000; Li *et al*., 2003). The efficacy of β-adrenergic modulation of cardiovascular function is decreased with aging, particularly during stress, and it has been thought that the age-associated deficit in the effectiveness of β-adrenergic control is largely postsynaptic, and that desensitization of β-adrenergic receptor (β-AR) responses occurs via modifications of both receptor and postreceptor events (Chevalier *et al*., 1991; Sakai *et al*., 1992; Lakatta, 1993; White *et al*., 1994; Esler *et al*., 1995; Ferrara *et al*., 1997; Hardouin *et al*., 1997). However, it was reported that neither G-protein-coupled-receptor kinases (GRKs) nor inhibitory G-proteins (Gi proteins) appear to contribute to the age-associated reduction in cardiac β-AR responsiveness (Xiao *et al*., 1998). The mechanism underlying this age-dependent reduction in cardiac β-adrenoceptor function is not completely understood. The norepinephrine transporter (NET) at presynaptic nerve terminals mediates the uptake of released norepinephrine (NE), resulting in the rapid termination of synaptic transmission and thereby controlling the fine tuning of neuronal activities (Esler *et al*., 1990; Povlock and Amara, 1997). Age-related reduction in reuptake of NE and β-AR density in the heart of health senescent human and animals was reported (Esler *et al*., 1990; 1995; Chevalier *et al*., 1991; Dawson and Meldrum, 1992; Sakai *et al*., 1992; White *et al*., 1994; Ferrara *et al*.,

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1997; Hardouin *et al*., 1997; Guimaraes *et al*., 1998; Snyder *et al*., 1998; Xiao *et al*., 1998). Failure of transmitter inactivation at postjunctional receptors with aging would amplify the neural signal. Because the myocardial interstitial NE content depends predominately upon the amount released and reuptake capacity by the neuron, a defective NE uptake could contribute to high concentration in the synaptic cleft and β-AR density downregulation, even in situations in which the release of NE from the sympathetic cardiac neurons is low (Esler *et al*., 1990; Povlock and Amara, 1997). Thus, it is speculated reasonably that the reuptake capacity of NET might have declined with age and contributed to β-AR downregulation and age-related decreases of cardiac β-adrenergic responsiveness. The results of radioactive scanning for cardiac sympathetic neuroimaging have generally agreed with the notion of an age-related decline in cardiac uptake activity (Leineweber *et al*., 2002; Kiyono *et al*., 2002; Kaye and Esler, 2005). However, there is little information regarding the speculation that changes of the upstream modulator of β-adrenergic responsiveness are involved in the age-related β-AR downregulation. Whether there are declined levels of both mRNA and protein of cardiac sympathetic NET with aging is not known at present. The aim of this investigation is to examine whether there is an age-related reduction in the cardiac NET and β_1-AR mRNA expressions.

MATERIALS AND METHODS

In this study, 2- (n=10) and 16-month-old (n=10) male Sprague-Dawley (SD) rats were used. Animal use procedures were followed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USA. After acclimatization period, rats were sacrificed by decapitation, and the middle and inferior (stellate) sympathetic ganglia (middle cervical-stellate ganglion (MC-SG) complex) of both sides were isolated. The sympathetic ganglionic innervation of rat heart originates primarily MC-SG complex (Pardini *et al*., 1989; 1990), and expression of cardiac NET mRNA is in the ganglia (Li *et al*., 2001).

The hearts (n=5, each age group) were dissected into the left and right ventricles, and the left ventricle was separated into two equivalent sections along its long-axis. The upper sections of left ventricular myocardium were quickly frozen in liquid nitrogen until the mRNA analysis performed. The lower sections of left ventricular myocardium were immersed and fixed with 0.04 g/ml paraformaldehyde at 4 °C, and the sections were cut into 18-μm thick slices for immunohistochemical staining for NET and adrenergic innervation of the heart.

The total RNA was extracted from the MC-SG complex and LV myocardium using the reagent of TRIzol (Gibco BRL, Gaithersburg, MD, USA) according to the instructions. DNase I was used to eliminate contamination of genomic DNA in RNA preparation, and 1 μg of total RNA of the ganglia was used for reverse transcription (RT) into cDNA with Moloney murine leukemia virus reverse transcriptase (M-MLV) (Gibco BRL) according to standard methods (Li *et al*., 2001).

NET and β_1-AR Northern blottings were performed as described previously (Li *et al*., 2001). Briefly, 15 μg of total RNA of the ganglia and left ventricular myocardia of 2- and 16-month-old rats were electrophresed on a 0.01 g/ml agarose-formaldehyde gel and transferred to a Hybond-N membrane (Amersham, International, Buckinghamshire, UK). The blots were prehybridised for 3 h and hybridised for 20 h with ^{32}P-labeled NET or β_1-AR cDNA as probe in 0.5 g/ml formamide at 42 °C. After autoradiography exposure, the blots were stripped and rehybridised with ^{32}P-labeled β-actin cDNA probe. Each hybridisation signal of specific band of NET and β_1-AR was measured by densitometry, and was normalized by the corresponding hybridisation signals obtained from the β-actin cDNA probe to correct differences in loading and transfer. The β-actin signal was used as a correction factor.

Probes were labeled by random priming with DNA labeling system (Boehringer Mannheim GmbH, Germany) and radioactivity of the probes at 1.8×10^9 cpm/μg. The NET probe is a 407-bp amplification product of the cloned rat NET gene (Kitayama *et al*., 1999) between a sense primer 5'-CTC AAG GAG GCC ACG GTA TGG ATC G-3' and an antisense primer 5'-ACC TGG AAG TCA TCA GCC AGT CCG G-3', and β-actin probe is a 285-bp product of the murine β-actin gene between a sense primer 5'-TCA TGA AGT GTG ACG TTG ACA TCC GTA

AAG-3' and an antisense primer 5'-CCT AGA AGC ATT TGC GGT GCA CGA TGG AGG-3' (Li *et al*., 2001). β_1-AR probe, a 900-bp *Pst*I coding fragment of the β_1-AR genomic clone, was a kind gift of Dr. CA Machida (Division of Neurosicence, Oregon Regional Primate Research Center, Oregon Health Sciences University, USA).

An avidin/biotin blocking kit (HISTOSTAIN™-DS Kit; Zymed Laboratories, San Francisco, CA, USA) was used for detection of NET expression. The sections of left ventricle were incubated in primary antisera:rabbit anti-NET (1:250 (v/v); Alpha Diagnostic Intl. Inc., San Antonio, USA) according to the manufacturer's instructions (Zymed Laboratories).

The immunohistochemical methods for tyrosine hydroxylase (TH) were used as previously described (Himura *et al*., 1993) with modification. Briefly, the specimens were soaked in methanol with H_2O_2 for 30 min. After washing in phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.2), the specimens were reacted with normal goat serum (1:20; Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Then, they were incubated with anti-TH antibody (1:100; Sigma Chemical, St. Louis, MO, USA) for 24 h at 4 °C. After rinsing with PBS containing 0.5 mg/ml Tween 20, they were incubated with secondary antibody (goat anti-mouse IgG, 1:100; Vector Laboratories) conjugated to horseradish peroxidase for 1 h. After rinsing with PBS containing 0.5 mg/ml Tween 20, they were reacted with avidin-biotin complex (ABC) reagent (Vector Laboratories). Finally, they were reacted for 5~10 min with 0.5 mg/ml 3,3′-diaminobenzidine (Sigma Chemical) and 0.01% (v/v) H_2O_2 in Tris buffer (pH 7.6). The sections were finally mounted on gelatin-coated slides, dried, dehydrated through a series of ethanol, cleared in xylene, and coverslipped in Permount.

These specimens were microscopically examined, and the distribution densities of the NET and TH of LV myocardium were calculated by the point-counting method (Kawano *et al*., 2003). Briefly, for each piece of myocardium, 10 fields were randomly selected in each area of a specimen, and a section was registered if the section contained the nerve fibers. The total counts of registered sections in the 10 fields were used to compare the distribution density of the nerves.

Values were expressed as mean±*SD*. Statistical analyses were performed by analysis of variance (ANOVA) followed by Dunnett's test or Student's *t*-test. Differences were considered significant at $P<0.05$.

RESULTS

To determine the differences of cardiac sympathetic NET and myocardial β_1-AR mRNA expression levels of LV between the 2- and 16-month-old rats, Northern blot was performed as previously described (Li *et al*., 2001). The NET mRNA and protein levels were reduced in 16-month-old rats by 67% (Fig.1) and 26% (Fig.2), respectively, and β_1-AR mRNA level in myocardia of the 16-month-old rats was reduced by 43% (Fig.3) in comparison with that in the ganglia ($P=0.001$) and that in myocardia ($P<0.05$, $P=0.018$) of 2-month-old rats, respectively. Meanwhile, about 51% reduction of the catecholaminergic nerve fiber density in the myocardia of 16-month-old rats was found (Fig.4), compared with 2-month-old rats ($P<0.05$).

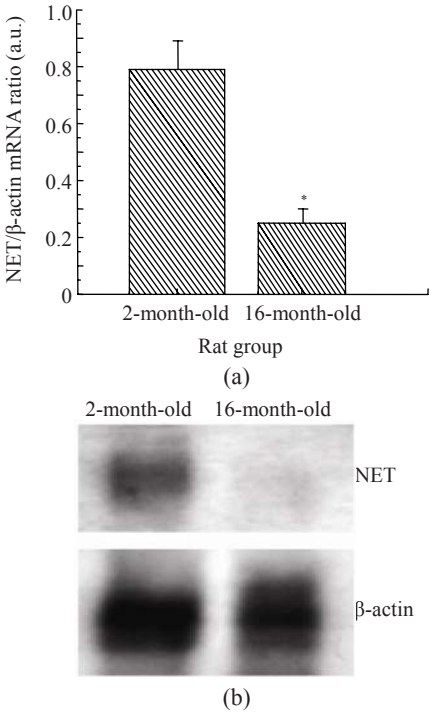

Fig.1 NET/β-actin mRNA levels in the MC-SG complex analyzed with Northern blot. (a) Quantitative analysis of NET mRNA; (b) NET mRNA detected by Northern blot in the MC-SG complex of rats of different ages

*$P<0.05$, 2-month-old rats vs 16-month-old rats; Mean±*SD*; $n=5$ for each group

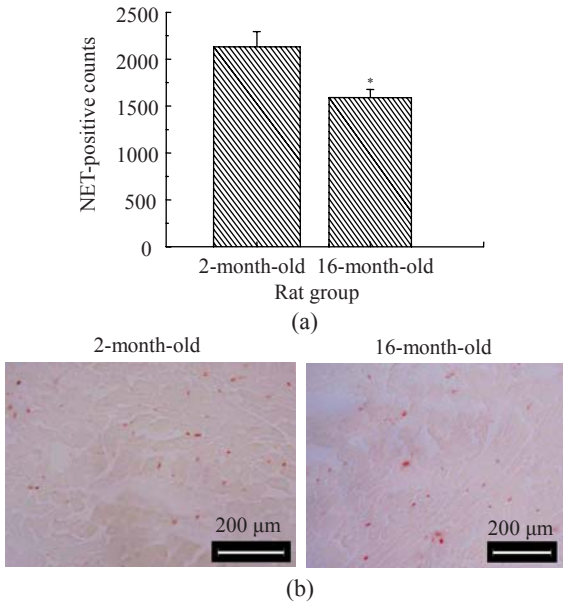

Fig.2 Comparison of myocardial NET positive counts between the 2- and 16-month-old rats. (a) Quantitative analysis of NET positive counts. Bar graph shows the mean count number of NET-positive sections by the point counting method in the left ventricle; (b) NET detected by immunohistochemistry in myocardia of rats of different ages

*P<0.05, 2-month-old rats vs 16-month-old rats; Mean±SD; n=5 for each group

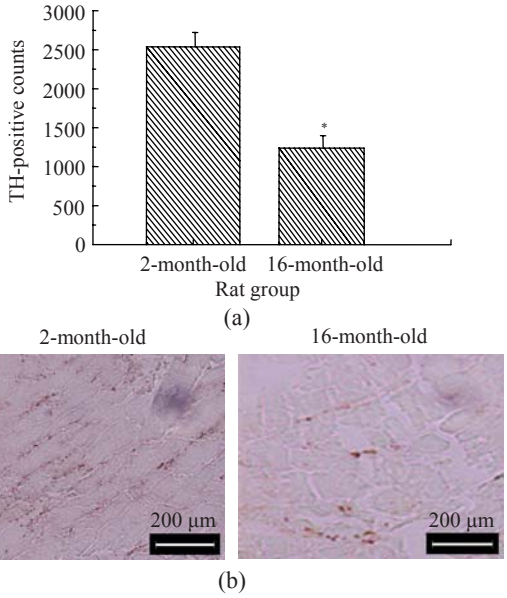

Fig.4 Comparison of the nerve distributions between the 2- and 16-month-old rats. (a) Quantitative analysis of TH positive counts. Bar graph shows the total number of nerve-positive sections in the myocardium of 2- and 16-month-old rats; (b) TH positive nerves detected by immunohistochemistry in myocardia of rats of different ages

*P<0.05, 2-month-old rats vs 16-month-old rats; Mean±SD; n=5 for each group

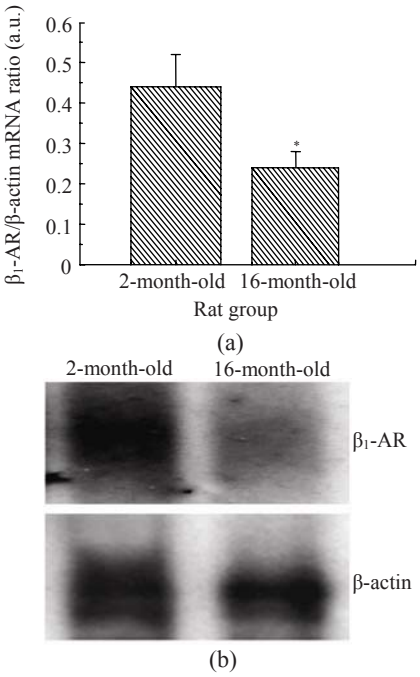

Fig.3 Myocardial β_1-AR to β-actin mRNA levels in 2- and 16-month-old rats analyzed with Northern blot. (a) Quantitative analysis of β_1-AR mRNA; (b) β_1-AR mRNA detected by Northern blot in myocardia of rats of different ages

*P<0.05, 2-month-old rats vs 16-month-old rats. Mean±SD; n=5 for each group

DISCUSSION

Sympathetic nerve system (SNS) regulates the function of innervated effector organs through releasing and terminating neurotransmitter NE (Esler *et al*., 1990; Francis, 1995). The released transmitters in the cleft bind receptors on the postsynsptic membrane through diffusion, transmitting the signals to the postsynaptic neurons. After the signal transmission is completed, the released neurotransmitters in the cleft must be rapidly eliminated from the cardiac synaptic cleft primarily via the NET on presynaptic membrane, so as to assure effectiveness of the nerve impulse and control the strength of the nerve impulse in this way (Barkely *et al*., 1994; Povlock and Amara, 1997). The signal transmission termination of majority of monoamines and amino-acids neurotransmitters predominantly depends on the reuptake of neurotransmitter transporters. Eisenhofer (1994) reported that 88% of released NE was removed by neuronal uptake, 4% was removed by extraneuronal uptake, and 8% escaped removal processes to spillover into

plasma. Only a small fraction of sympathetic cleft NE gains access to the circulation, so that changes in transport or changes in reuptake could have a profound effect on the circulating levels. NET controls NE concentration in the cleft and around β-AR (Esler *et al*., 1990; Barkely *et al*., 1994; Eisenhofer, 1994; Povlock and Amara, 1997). It has long been recognized that prolonged exposure of myocardial tissue to β-adrenergic agonists modifies the β-AR responsiveness, and that densensitization of β-AR stimulation occurs via modifications of both receptor and postreceptor events. But evidence has accumulated that dysfunction of cardiac reuptake of NE leads to reduction of reuptake of NE and myocardial β_1-AR with aging (Chevalier *et al*., 1991; Dawson and Meldrum, 1992; Sakai *et al*., 1992; Lakatta, 1993; White *et al*., 1994; Esler *et al*., 1995; Ferrara *et al*., 1997; Hardouin *et al*., 1997; Guimaraes *et al*., 1998; Snyder *et al*., 1998; Li *et al*., 2003). Previous experimental results showed that NET mRNA was exclusively expressed in noradrenergic cells of the cardiac ganglia rather than in the myocardia (Ungerer *et al*., 1996; Bohm *et al*., 1998; Backs *et al*., 2001; Li *et al*., 2001), and was strictly localized to the perikarya but not detected in nerve fibers projecting from the ganglia (Backs *et al*., 2001). The sympathetic origin of the cells stained positively for NET mRNA was specified by demonstration of TH immunofluorescence (Backs *et al*., 2001). The results suggest that the NET proteins colocalized in the sympathetic nerve are transported from a distance from the heart; therefore it might also be considered as a marker of the noradrenergic nerve. But the distribution of NET proteins localized along the fibers in the myocardium may not be consistent with the TH positive fiber density. Recently, Wehrwein *et al*.(2008) reported that the NET protein is colocalized in nerve fibers with TH immunoreactivity in murine atria; however, the NET protein does not correlate with innervation density of murine heart after normalizing to the total protein content of each of the chambers. Rather, NET appears to be equally distributed throughout the myocardium; the total NET protein was highest in the ventricles and lowest in the atria (Wright *et al*., 2006; Wehrwein *et al*., 2008). In contrast, postsynaptic effector, β-AR, is uniformly distributed throughout the myocardium, with similar densities in both atrial and ventricular tissues (Hata *et al*., 2004).

The primary finding of present study is that age-related decline of NET levels of both mRNA and protein, together with the pattern of β_1-AR mRNA expression, confirms the previous findings (Sakai *et al*., 1992; White *et al*., 1994; Ferrara *et al*., 1997). They showed that myocardium decreased with aging, which is similar to cardiac sympathetic NET. Our results suggest that NET might contribute to the decrease in myocardial β_1-AR mRNA with aging. The NET change appears accordant with previous observations (Shores *et al*., 1999; Zhu *et al*., 2005), which showed that NET mRNA expression in locus coeruleus (LC) was significantly decreased with aging in rats, and the level of NET in the LC of 23-month-old rats showed less than half of that found in the 1-month-old rats (Zhu *et al*., 2005). These previous findings also indicate that there was an age-dependent decrease in myocardial NET binding sites by 36% in elderly patients [(60±3) years] without apparent heart failure, and NET activity was ~50% lower than that in children [(14±3) years] (Leineweber *et al*., 2002). However, the present results showed that age-related reduction of NET mRNA appears not parallel to its protein expression level. This could be due to an increase in the rate of NET protein production or a decrease in NET protein removal, or both. Recent studies demonstrated that the ratios of protein levels do not have a one-to-one correlation with the ratios of the corresponding mRNAs, which suggests that the relation between transcription and translation, consequently between mRNA and protein, is complex (Ideker *et al*., 2001; Mehra *et al*., 2003).

There is evidence indicating that the maximum rate of NE uptake (i.e., V_{max} for uptake [^{3}H]NE) declined significantly and the number of NET per synaptosome may decline with age and decrease in β-AR density in rat heart (Snyder *et al*., 1998). The reduction of the cardiac response to the sympathetic stimuli during aging may be partly explained by a decrease in the corresponding receptor density; the changes are reversible and the density of the receptors can return to adult control values by chronic administration of the appropriate antagonist (Chevalier *et al*., 1991). Similarly, clinical studies showed that reduced NE reuptake increases the overflow of the neurotransmitter to plasma from the aging heart during stimulation of the cardiac sympathetic outflow (Esler *et al*., 1995) and β-AR downregulation in the hearts of old

subjects (White *et al*., 1994). Furthermore, it is well documented that NET, the cardiac local modulator, is a major factor contributing to downregulation of myocardial β-AR density in heart failures of both human and animal models (Liang *et al*., 1989; Bristow *et al*., 1992). The important role of NET in terminating the action of released NE contributes to the maintenance of NE levels in the nerve terminals as well as to rapid regulation of NE levels around the receptors, which in turn influences the expression of post synaptic β-AR.

In the present study, the changes of mRNA and protein levels suggested the lower transcription rather than NET internalization in the hearts of the aged rats, because changes in cell surface expression of transporter protein measured with antibody-based techniques, such as immunohistochemical and Western blot analyses, may not be detected. These techniques usually, but not always, measure the total number of transporters in the cell (Zahniser and Doolen, 2001). Therefore, it is reasonable that NET protein biosynthesis reduces during aging, which likely contributes to the reduced cardiac reuptake capacity of NE. Since NET is a component of sympathetic nerves, the recent report by Bruzzone *et al*.(2003) of an age-related decrease of the sympathetic is consistent with the present finding of a lower NET density with age. The reduction in NET protein might also be at least partly due to a loss of the nerve terminals, even though the NET protein does not correlate with the innervation density in myocardium (Wright *et al*., 2006; Wehrwein *et al*., 2008). The finding suggests that the upstream modulator of β-AR, cardiac symapthetic NET, may have a predominant role in age-related density downregulation of myocardial β_1-AR in senescent rats, which may contribute to both of β-AR downregulation and age-related decrease of cardiac β-adrenergic responsiveness.

However, the mechanism responsible for downregulation of cardiac NET with aging is not clear at present; the reduction in mRNA could be due to a decrease in transcription of the gene and/or a change in mRNA stability. It is known that DNA binding activity of activating protein-1 transcription factor decreases with aging (Asanuma *et al*., 1995; Tumer *et al*., 1997), which could contribute to a lower transcription rate of the adenosine A-1 receptor (A1-AR) gene. Whether this is relevant to the expression of NET mRNA is not clear and will need further investigation. Moreover, there are an accumulating pool of evidence that the central neural mechanisms involved the increase of cardiac sympathetic nerve activity (Aggarwal *et al*., 2002; Rosen *et al*., 2004; Kaye and Esler, 2005; Leenen, 2007), so we conclude that alteration of central nervous system activity deteriorates significantly with aging, which might be implicated in the function of cardiac sympathetic nerve system.

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