Age-related regional differences in cardiac nerve growth factor expression

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Abstract Age has been identified as an independent risk factor for cardiovascular diseases. A shift of the cardiac autonomic nervous system towards an increase in sympathetic tone has been reported in the elderly. Nerve growth factor (NGF) is the main neurotrophic factor that increases the sympathetic activity of the heart. If there is a shift of NGF expression in old compared to young cardiomyocytes and whether there are regional differences in the heart still remain unclear. Therefore, we chose a rat model of different-aged rats (3–4 days=neonatal, 6–8 weeks=young, 20–24 months=old), and isolated cardiomyocytes from

the left and the right atrium (LA, RA), as well as from the left and the right ventricle (LV, RV), were used to determine NGF expression on mRNA and protein levels. In neonatal, young, and old rats, NGF amount in LA and RA was significantly lower as compared to LV and RV. In young and old rats, we found significant higher NGF protein levels in LA compared to RA. In addition, both atria showed an increase in NGF expression between age groups neonatal, young, and old. In both ventricles, we observed a significant decrease in NGF expression from neonatal to young rats and a significant increase from young to old rats. The highest NGF amount in LV and RV was observed in neonatal rats. Regarding tyrosine kinase A receptor (TrkA) expression, the main receptor for NGF signaling, both atria showed the largest expression in old rats; while in LV and RV, TrkA was expressed mainly in young rats. These results point to a contribution of nerve growth factors to the change of autonomic tone observed in elderly patients.

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Abbreviations

NGF	Nerve growth factor
TrkA	Tyrosine kinase A receptor
NRCM	Neonatal rat atrial and ventricular myocytes
N	Neonatal rats (3–4 days)
Y	Young rats (6–8 weeks)
O	Old rats (20–24 months)

RA	Right atrium
LA	Left atrium
RV	Right ventricle
LV	Left ventricle

Introduction

Age is an independent risk factor for cardiovascular diseases (Boettger et al. 2010). With rising age, the incidence of cardiovascular diseases like arterial hypertension, atherosclerosis, and myocardial infarction (MI) increases significantly (Franklin et al. 1997; Kannel et al. 1979). Since MI is one of the most common causes of chronic heart failure (CHF), the prevalence of CHF also increases with age (Roger et al. 2011). Likewise, age is accompanied by an increased sympathetic tone, which negatively affects all these age-related cardiovascular diseases. Although there is huge evidence for an increased sympathetic drive in the elderly on the functional level (e.g., heart rate, rhythm and heart rate variability), little is known about age-related changes of neural growth and cellular neural function in the aging myocardium.

Nerve growth factor (NGF) is the main neurotrophic factor that crucially controls the sympathetic tone of the mammalian heart (Levi-Montalcini 1976; Snider 1994; Hassankhani et al. 1995). In parallel, NGF has been identified as a pro-survival factor for cardiomyocytes by developing anti-apoptotic effects via tyrosine kinase A (TrkA) receptor downstream signaling (Caporali et al. 2008). With regard to its pivotal role in the integrity of cellular and subcellular systems, the autonomic nervous system has been further shown to be involved in heart rate, rhythm, and contractility (Wasmund et al. 2003; Meyer et al. 2010).

However, a shift of cardiac NGF expression in relation to age is largely unknown. Therefore, this study aimed to investigate age-related regional differences of cardiac NGF expression in different-aged rats.

Materials and methods

Animals

All animal experiments were approved by the local Ethics in Animal Research Committee, University

RWTH Aachen, Germany (TV-No.: 10596 A). The investigation also conforms to the Guide for Care and Use of Laboratory Animals US (NIH Publication No. 85–23, revised 1996). The 3–4-day (neonatal rats = N), 6–8-week (young rats = Y), and 20–24-month (old rats = O)-old male Sprague Dawley rats were obtained from Charles River (Germany). “n” represents the number of cell preparations. Neonatal rat atrial and ventricular myocytes (NRCM) were isolated and cultured as described previously (Rana et al. 2009; Saygili et al. 2009). Each NRCM cell preparation included 25–30 neonatal rats. Briefly, all four heart chambers were excised, and the pieces were subjected to an enzymatic and mechanical procedure. Neonatal myocytes were separated from fibrocytes through a Percoll gradient passage. Young and old male rats were euthanized with isoflurane prior to experiments as described previously (Rana et al. 2010b). Each young and old rat cell preparation included two to three rats from the same age group. Hearts were rapidly excised and arrested in ice-cold Krebs buffer (composition in mmol/L: NaCl 133, KCl 5, MgCl₂ 2, KH₂PO₄ 1.2, CaCl₂ 0.2, taurine 6, creatine H 6, glucose 10, HEPES 10, pH 7.4). The hearts were cannulated via the ascending aorta for retrograde perfusion. Hearts were perfused with Krebs buffer constantly gassed with 100% O₂ at 37°C with a constant pressure of 40 mmHg in the non-recirculating Langendorff mode for 15 min. Thereafter, the hearts were perfused with Krebs buffer containing an enzyme solution (200 mg/L collagenase, 280–300 U, Worthington, USA) and 1% BSA for 5–15 min. Both atria and ventricles were excised, and by gentle pipetting, cells were released from tissue chunks. The resulting cell suspension was filtered through a 200-μm-nylon mesh, and the cells were immediately prepared for protein and mRNA samples.

Immunofluorescence (IF) experiments

Hearts were fixed in 4% formaldehyde. From the paraffinized tissue blocks, 2-μm-thick sections were taken, mounted on charged slides, and stained as described below. Sections were deparaffinized and rehydrated through a graded ethanol series. Sections were incubated at room temperature with primary antibodies for 2 h, then with a secondary antibody

coupled to Alexa Fluor 488 (Invitrogen). To visualize fluorescence signals, the Axiovert-200 M microscope and the AxioVision Rel.4.5 software from Zeiss were used as described previously (Saygili et al. 2009).

Antibodies

The following primary antibodies were used: rabbit anti-NGF (Santa Cruz, sc-548), goat anti-TrkA (R&D Systems, AF175), rabbit anti-GAPDH (Cell Signaling, #2118), mouse anti-PGP9.5 (Abcam, ab20559), rabbit anti-neurofilament (Santa Cruz, sc-25652), and mouse anti-endothelin-1 (Acris, SM5063).

RNA preparation, first-strand cDNA synthesis, and quantitative real-time reverse-transcription PCR

RNA extraction, first-strand cDNA synthesis, and quantitative real-time reverse-transcription PCR were performed as described previously (Saygili et al. 2007). PCR primers and fluorogenic probes were purchased from Applied Biosystems (Foster City, CA). The assay numbers were as follows: Rn00560865_m1 (beta-2 microglobulin), Rn01533872_m1 (NGFb), and Rn00572130_m1 (TrkA). All PCR data are derived from at least $n=3$ cell preparations for each age. NGF expression in all heart chambers were normalized to LA.

Western blot

Western blots (WB) were performed as described previously (Saygili et al. 2009). All WB data are derived from at least $n=3$ cell preparations for each age.

Statistical analysis

All values are expressed as mean $\pm$ SEM. Comparisons between paired variables were performed using the Student's t test with a significance level of $P<0.05$.

Results

Regional differences in NGF mRNA expression

We first examined the mRNA expression of NGF in both atria and ventricles from N, Y, and O rats. Figure 1a shows the differences in NGF mRNA expression in all four heart chambers normalized to

LA within one age group. In N rats, the NGF mRNA expression in both ventricles is almost 16-fold higher as compared to both atria. In Y rats, the NGF mRNA expression in both ventricles is almost 8-fold higher as compared to both atria. In O rats, the NGF mRNA expression in both ventricles is almost 12-fold higher as compared to the according atria. Within one age group, there were no significant differences in NGF gene expression between LA and RA or LV and RV.

Regional differences in NGF protein expression

With Western blotting, we could confirm the gene expression data. We analyzed the NGF protein expression in all four heart chambers from N, Y, and O rats. Figure 1b shows that both LV and RV express approximately 4-fold higher NGF levels as compared to LA and RA. In Y and O rats, we observed higher NGF levels in LA as compared to RA.

NGF protein expression depends on age

First, we analyzed the NGF protein expression in both atria in relation to age. Both atria showed an increase in NGF expression between age groups N, Y, and O (Fig. 2a). In the next step, we investigated the influence of age on NGF expression in LV and RV. In both ventricles, we observed a significant decrease in NGF expression between age groups N to Y, and in turn, a significant increase from Y to O rats. The highest NGF amount in LV and RV was observed in N rats (Fig. 2b).

Visualization of NGF by IF experiments

To visualize NGF in heart tissue, we performed IF experiments as described in the “Materials and methods” section. Figure 3 shows representative images of NGF allocation in N rats in all four heart chambers. The highest NGF content was observed in LV and RV. Within LV and RV, NGF sources are especially localized near the endocardium, whereas the epicardium shows only a low degree of NGF expression.

Age-related TrkA expression in rat hearts

Regarding the pivotal role of NGF in cardiomyocyte cell apoptosis, which is mediated via its high affinity receptor TrkA, we investigated the expression of TrkA on mRNA and protein levels in all four heart

Fig. 1 Regional differences in NGF expression. **a** NGF gene expression analysis by real-time PCR shows that both ventricles express more than 10-fold higher mRNA levels as compared to both atria. **b** Western blots additionally reveal the significant difference in LA to RA NGF protein expression in Y and O rats

(N=3–4 days, Y=6–8 weeks, O=20–24 months; N neonatal, Y young, O old). All PCR and WB data are derived from at least $n=3$ cell preparations. NGF expression in all heart chambers were normalized to LA. * $P<0.05$ vs. LA. # $P<0.05$ vs. RA

chambers (LA, RA, LV, and RV). We found that the amount of TrkA in LA and RA of Y rats was significantly lower than in N rats. The highest amount

of TrkA in the atria was observed in O rats. These data reflect both the mRNA and protein levels (Fig. 4a–c). On the other hand, TrkA expression in

Fig. 2 Age-dependent differences in NGF expression. **a** Western blots additionally revealed a significant difference in LA compared to RA in Y and O rats. **b** In both ventricles, NGF expression significantly decreased from N to Y and increased in return from Y to O rats. The highest NGF amount in LV and RV

was observed in N rats (N=3–4 days, Y=6–8 weeks, O=20–24 months; N neonatal, Y young, O old). All WB data are derived from at least $n=3$ cell preparations. NGF expression in all age were normalized to N. * $P<0.05$ vs. N. # $P<0.05$ vs. Y

Green = NGF

Fig. 3 Representative immunofluorescence images showing the allocation of NGF protein in all four heart chambers of N rats. While LA and RA show only a very low degree of NGF, LV and RV show abundance of NGF levels. In LV and RV, higher NGF levels are localized near the endocardium, whereas the epicardium shows only a low degree of NGF expression

LV and RV showed the greatest amplitude in Y rats. In O rats, the boost of TrkA expression that was observed in Y rats depleted to control levels (Fig. 4e, f). The ventricular TrkA mRNA expression in Y rats showed an almost 35-fold augmentation in LV and an approximately 11-fold increase in RV as compared to N rats (Fig. 4d). On protein level, we observed a nearly 5-fold increase in LV and an approximately 3.5-fold increase in RV.

Age-related endothelin-1 expression in rat hearts

Endothelin-1 (ET-1) has been shown to regulate the expression of NGF in the rodent heart (Ieda et al. 2004), and its endogenous expression is required for cardiomyocyte survival (Zhao et al. 2006). In the present study, we found that the expression of ET-1 in LA and RA positively correlated with the expression of NGF, with respect to age. Both atria showed an increase in ET-1 expression from N to Y and O rats

(Fig. 5a). By contrast, in RV and LV, a rise of ET-1 expression was present from N to Y but no further increase to O rats (Fig. 5b).

A neuronal contamination can be excluded

To rule out a neuronal contamination during the cardiomyocyte cell isolation procedure, we further performed WB experiments with the nonspecific neuronal markers PGP9.5 and neurofilament (NF). Figure 6 shows that there is no expression of PGP9.5 or NF in the WB samples.

Discussion

The major findings of the present study are: (1) Through all age groups (N, Y, and O), both ventricles expressed almost 4-fold higher NGF protein levels as compared to the atria. (2) Both atria showed a continuous increase of NGF expression from N to O rats. (3) There was a decrease of the ventricular NGF content from N to Y rats followed by an increase from Y to O rats. (4) Maximum TrkA expression in the atria was observed in O rats, whereas ventricular TrkA expression was highest in Y rats. (5) The expression of ET-1 correlated with the expression of NGF in LA and RA, while LV and RV ET-1 expression increased between age groups N and Y rats with no relevant differences between age groups Y and O rats.

The results of this observational study will have to be interpreted cautiously and may rather serve as basis for a new hypothesis. In general, an increase of NGF expression can be observed both in atria and ventricles. Since NGF also exerts anti-apoptotic effects via its main receptor TrkA (Caporali et al. 2008), the observed NGF increase may in fact represent a reflex mechanism to an increased degree of apoptosis in aging myocardium. Since apoptosis and fibrosis in the atria and even in healthy hearts seem to develop with age (Olivetti et al. 1997; Centurione et al. 2002), the continuous increase of NGF may be in line with such a hypothesis. Such a mechanism, however, would not explain the decrease of NGF expression in the ventricles from N to Y rats. Since NGF is also required for the differentiation of immature neurons, this effect may be predominant in N ventricles and may then fade in Y

Fig. 4 Age-dependent differences in TrkA receptor expression. In both atria, the highest levels in TrkA expression were observed in O rats. These data reflect both the mRNA and protein expression (a–c). Regarding the ventricles, TrkA mRNA levels showed the highest degree in Y rats, with an almost 35-fold augmentation in LV and an approximately 11-fold increase in RV (d). On protein level, a nearly 5-fold

increase in LV and an approximately 3.5-fold increase in RV were detected. In O rats, the boost of TrkA expression that was observed in Y rats depleted to control levels (e–f; N=3–4 days, Y=6–8 weeks, O=20–24-month rats; N neonatal, Y young, O old). All PCR and WB data are derived from at least $n=3$ cell preparations. NGF expression in all groups were normalized to N. * $P<0.05$ vs. N. # $P<0.05$ vs. Y

rats before a potential anti-apoptotic mechanism starts to be operative.

NGF exerts its anti-apoptotic effects via TrkA receptor signaling. In our study, we observed an increase of TrkA with age in the atria but a decrease from Y to O in the ventricles. Thus, the observed decrease of TrkA in the ventricles in O rats fits well to the parallel increase of NGF. Likewise, a downregulation of ET-1 expression may reflect a negative feedback loop of increased NGF levels since ET-1 has been shown to regulate the expression of NGF in the rodent heart (Ieda et al. 2004). The concordant course of NGF and TrkA in the atria, by contrast, may point to a differential and probably multifactorial regulation of TrkA expression in the atria by yet unknown additional contributors. An alternative mechanism might be an agonist-dependant upregulation of TrkA receptors in the atria, which, so far however, has not been described.

The role of NGF in heart diseases has been thoroughly reviewed by Govoni and colleagues (Govoni et al. 2011). NGF plays a pivotal role in sympathetic

nerve sprouting and sudden cardiac death (Chen et al. 2001). An early rise of NGF can be observed rapidly after myocardial infarction (hours) and may possibly reflect an early rescue response to support myocardial healing (Meloni et al. 2010). Unfortunately, NGF also causes inhomogeneity of sympathetic innervation in the infarction border zone thereby promoting lethal arrhythmias (Zhou et al. 2004). Months later, if heart failure develops after MI, the opposite is detected and NGF tissue levels decrease below the normal range. Reason for this may be multifold. During chronic heart failure, myocytes are exposed to increasing wall stress, which has been shown to decrease the NGF expression in cardiomyocytes, sufficient to reduce sympathetic nerve growth (Rana et al. 2009). In parallel, during chronic heart failure, there is an overflow of norepinephrine, which contributes to a downregulation of NGF in cardiomyocytes (Hasking et al. 1986; Kimura et al. 2010). Likewise NGF expression was shown to decrease in response to α -1 receptor stimulation (Rana et al. 2009). By contrast, sympathetic neurons

Fig. 5 The expression of ET-1 shows a positive correlation to NGF regarding both atria and differs to both ventricles. Both atria showed an increase in ET-1 expression between age groups N (neonatal), Y (young), and O (old) rats (**a**). On the other hand, regarding LV and RV, Y rats showed significantly

higher ET-1 levels than N rats. No significant differences were found between the groups Y and O rats (**b**). All WB data are derived from at least $n=3$ cell preparations. ET-1 expression in all groups were normalized to N. * $P \leq 0.05$ vs. N. # $P \leq 0.05$ vs. Y

respond to mechanical stretch with an upregulation of NGF that results in increased sympathetic nerve growth (Rana et al. 2010a). The fine interplay between these two cellular systems (myocytes and neurons) under physiological or pathological circumstances, however, is not well understood.

The present study raises the opportunity that an age-associated increase of NGF levels may promote the development of sympathetic hyperinnervation in the aging heart. Such a mechanism would be in line with the observation of sympathetic nerve sprouting in AF, which, so far was regarded as a consequence of AF. In

light of the results of the present study, sympathetic nerve sprouting in AF might also reflect age-related changes of the atria since AF is a typical age-related disease.

Limitations

In the present study, we examined rat hearts to characterize the expression of NGF, TrkA, and ET-1 with respect to age. Thus, the expression patterns in our model may differ substantially from those of human. In addition, NGF is a target-derived protein with neuronal and non-neuronal sources. The present study examined the expression of NGF only in isolated cardiomyocytes. Furthermore, we cannot report on whether the upregulation of NGF in atria with age exerts a positive feedback mechanism on the expression of TrkA, since both seem to increase with age. Despite the knowledge that ET-1 regulates the expression of NGF in the rodent heart, our data could show only a correlation for these proteins in the atria, whereas the expression in the ventricles developed not in parallel with age. In addition, we did not analyze the expression of the ET-A receptor, which is mainly responsible for ET-1 signaling in cardiomyocytes.

Fig. 6 Neuronal contamination can be excluded. With nonspecific neuronal markers (PGP9.5 and neurofilament), a possible neuronal contamination that might have taken place during the cell isolation procedure could be excluded

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