Ageing-related aorta remodelling and calcification occur earlier and progress more severely in rats with spontaneous hypertension

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Summary. The effects of hypertension on vascular remodelling, ageing and calcification are not fully understood. In this study, we monitored the dynamic changes of aorta remodelling, senescence and calcification in spontaneously hypertensive rats (SHRs) during ageing. Results. Vascular remodelling and senescence cells occurred in SHR aortas at 24 weeks. The calcium content and calcium deposition of the aorta increased in SHRs at 48 weeks. All of these changes became increasingly significant with ageing. In contrast, these pathologic changes appeared in Wistar-Kyoto (WKY) normotensive rats at a later stage (72 weeks). These data showed that the ageing-related vascular remodelling, senescence and calcification in SHRs occurred earlier and progressed more severely than in WKY rats. Conclusion. Ageing-related vascular remodelling and calcification were accelerated and augmented in SHR aortas.

Key words: Hypertension, Vascular ageing, Vascular remodelling, Vascular calcification

Introduction

Ageing is the primary independent risk factor for cardiovascular disease (Lakatta and Levy, 2003). The direct relationship between ageing and vascular health is extremely evident in early ageing syndromes; patients experience accelerated ageing and premature death due to cardiovascular and cerebrovascular complications, such as stroke and myocardial infarction (Gerhard-Herman et al., 2012). Vascular ageing is one of the major ageing manifestations and important initial factors, as well as the basis of various ageing-related diseases. Age related vascular calcification is a degenerative change that occurs during ageing, as well as one of the important symptoms and mechanisms of vascular ageing. Vascular calcification is very common in older people, causing decreased arterial elasticity and increased morbidity and mortality in cardiovascular disease (Demer and Tintut, 2008; Zhu et al., 2012; El Asmar et al., 2014).

Vascular calcification includes intimal atherosclerotic calcification, arterial media calcification (Mönckeberg's arteriosclerosis), cardiac valve calcification, end-stage renal disease vascular calcification and calcific uremic arteriolopathy. Arterial media calcification belongs to the ageing related type of vascular calcification (Demer and Tintut, 2008). Currently, the reasons and mechanisms for age related vascular calcification are not fully understood, and its relationship with hypertension has not been elucidated (Rattazzi et al., 2012). In addition, the results of some previous studies using animal models were contradictory. For example, Kieffer et al. (2000) showed that hypertension did not affect the vascular calcium content of aged rats; but Fleckenstein et al. (1990) found that the arterial calcium content of SHRs was significantly higher than the age-matched WKY normotensive rats. The differences in these results could be due to different experimental methods and may also relate to the lack of investigation into the dynamic
changes of vascular calcification during ageing. Therefore, in this study, we investigated the dynamic changes of aorta remodelling, ageing and calcification during SHR ageing (4 weeks to 72 weeks old) and compared them with age-matched WKY rats.

Materials and methods

Animals

The specific pathogen free (SPF) male SHRs and WKY rats at 3 weeks old were purchased from Shanghai Slack Experimental Animal Company (License No: SCXK (Shanghai) 2012-0002). The rats were raised in separate cages in the SPF facility. The light was manually controlled (daylight 12 h, dark 12 h). The temperature was kept at 22-24°C, and the relative humidity was 45-55%. The rats were fed a standard diet with no food or water restrictions. All animal studies were conducted in compliance with the national “Laboratory Animals Regulations” and “Fujian Regulations of Laboratory Animal Management” and approved by the Institutional Animal Care and Use Committee of Fujian Medical University (Fuzhou, Fujian, China).

Methods

Experimental groups

After one week of adaptive feeding, the SHR rats were randomly divided into the following 5 groups (n=10 for each group): 4 weeks old group (4 w, juvenile), 12 weeks old group (12 w, adolescence), 24 weeks old group (24 w, adult), 48 weeks old group (48 w, adult) and 72 weeks old group (72 w, aged). Age-matched WKY rats were used as a control group.

Measurement of systolic blood pressure

Systolic blood pressure (SBP, mmHg) was measured with the noninvasive blood pressure metre BP-2010A (Softron Beijing Incorporated, Beijing, China) according to the Tail-Cuff method.

Tissue sample collection

The rats were fasted for 24 h and then given 2.5% pentobarbital sodium (80 mg/kg) for anaesthesia via intraperitoneal injection after being weighed. Rats were perfused under pressure with 0.1 M PBS (pH 7.4 at 25°C) for 30 minutes via the left ventricular. Pressure was fixed at the level corresponding to central aortic mean pressure, calculated from tail artery systolic pressure (Amin et al., 1996). Then, the total length of the thoracic aorta (from the lower edge of the fourth thoracic vertebra, which is connected to the aortic arch to the aortic hiatus in the diaphragm) was excised. Approximately 5 mm of the thoracic aorta at the proximal end was rinsed with PBS and immediately placed in freshly prepared 4% paraformaldehyde/0.1 M PBS (pH 7.0-7.6) for 24 h (at 4°C). The 1-2 mm at the proximal end was used for frozen sections (thickness 8 μm), which were then used for senescence associated-β-galactosidase (SA-β-Gal) staining. The latter 3-4 mm was used for continuous paraffin sections (thickness 5 μm) with the proximal end facing up. The remaining thoracic aorta was stored in liquid nitrogen for a subsequent western blot assay and calcium content measurement.

Vascular morphology staining

The first 1-3 continuous paraffin sections of each vessel were used for haematoxylin-eosin (HE) staining (Lin et al., 2015), the 4-6th sections were used for Elastic Van Gieson (EVG) staining (Lin et al., 2015), and the 7-9th sections were used for α-smooth muscle actin (α-SMA) immunofluorescence staining (Lin et al., 2015).

EVG staining was used to show elastic fibre and collagen fibres. Elastic fibres are blue under a microscope, while collagen fibres are pink. EVG staining was performed following the kit instructions (BATR-4083, Beijing Zhongshan Jinqiao Biotechnology Inc.) The results were collected and analysed with Image-Pro Plus 6.0 software (Media Cybernetics Inc, Rockville, MD). The media thickness (MT), luminal diameter (LD), MT/LD ratio, media area (MA) and the percentage of media collagen fibres were measured and calculated.

Measurements of α-SMA expression

Immunofluorescence staining: Paraffin sections were dewaxed and hydrated, followed by high pressure antigen retrieval for 2 min. Then, the sections were incubated with 3% H2O2 for 10 min, washed with PBS and blocked with goat serum for 30 min; then, the serum was removed, and α-SMA primary antibodies (1:2000, Sigma, USA) were added and incubated overnight at 4°C. PBS was used as a negative control for primary antibodies. After the primary antibody was washed by PBS, the fluorescent secondary antibody (rhodamine labelled goat anti-mouse IgG, dilution 1:50) was added and incubated at room temperature for 90 min in the dark. Then, the sections were washed with PBS and incubated with tissue autofluorescence quencher at room temperature in the dark for 90 min. The sections were mounted with anti-fluorescence decay mounting media. Red fluorescence indicated a positive signal. DAPI staining was used to visualize the cell nucleus to normalize the relative expression of α-SMA. All the images were acquired using the same exposure parameters.

Western blot: The rat aorta was pulverized in liquid nitrogen, and the protein was extracted and quantified. A total of 20 μg of protein was loaded for SDS-PAGE, followed by transferring to a PVDF membrane. The
membrane was blocked with 5% nonfat milk for 2 hours at room temperature, and incubated with primary antibody overnight at 4°C. The following primary antibodies were used: mouse monoclonal anti-α-SMA antibody (1:1000, Sigma, USA) and rabbit polyclonal anti-β-actin antibody (1:5000, Abcam, UK). After incubation with appropriate goat anti-rabbit (1:2000 dilution) or goat anti-mouse (1:2000 dilution) secondary antibodies conjugated with HRP (Millipore, USA) for 1 h, ECL-HRP luminescent substrate was applied on the membrane for 1 min, and the X-ray film was imaged with a gel imaging system. The bands were analysed and quantified by Quantity One image analysis software. The protein expression level is denoted as the ratio between the intensity of the target band and the intensity of the β-Actin band.

SA-β-Gal staining (Dimri et al., 1995)

Frozen sections were dried at room temperature and fixed with 4% paraformaldehyde/0.1 M PBS (pH 7.0-7.6) for 10 mins. After washing with PBS, the slides were placed in a wet box and incubated with freshly prepared SA-β-Gal incubation buffer (5-bromo-4-chloro-3-indole-β-D-galactoside (X-Gal, Sigma, USA) 1 mg/ml, 40 mmol/L citrate buffer, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L sodium chloride, 2 mmol/L magnesium chloride, pH 6.0) at 37°C for 20 hours in the dark. Then, the staining buffer was washed off by gentle running water, and the sections were counterstained with eosin. Finally, the sections were washed with tap water, baked and mounted with neutral gum, followed by microscopic examination. The negative control sections were incubated with SA-β-Gal working solution without X-Gal, and the positive control sections were incubated with SA-β-Gal working solution at pH 4.0. The senescent cells were stained indigo blue. Under the microscope, three fields were selected randomly clockwise on each slide, and the percentage of positive coloured areas in the tunica media of the observed field were measured and averaged.

Aortic calcium content measurement (Wu et al., 2003; Neven et al., 2015)

The aorta tissue was placed in a small culture dish, rinsed twice with ultra-pure water, dried in the drying oven for 1 h (80°C), and then weighed after cooling down to room temperature. After that, the tissue was supplemented with 4 mL 2 mol/L concentrated nitric acid and digested with microwave (temperature 150°C, pressure 15 atm) for 5 min. After being cooled down, the tissue was supplemented with 10 mL ultra-pure water (containing 27 nmol/L potassium chloride and 27 μmol/L lanthanum chloride) for redissolution; 2 mL of the sample was taken and added with 100 μL 1 % (mass fraction) strontium chloride. Then, the absorbance of each tube at 422.7 nm wavelength (lamp current 3 mA, burner height 6 mm, gas flow 1700 mL) was measured using an atomic absorption spectrophotometer (TAS-986, Beijing General Analytical Instruments Company), and the unit was converted to μmol/g.wt.

von Kossa staining (Wu et al., 2003; Neven et al., 2015)

The 10-12th continuous paraffin sections were used for von Kossa staining. Sections were dewaxed and hydrated and then stained with 5% silver nitrate solution, followed by irradiation with a UV lamp for 60 min. Then, the sections were washed with distilled water, treated with 5% sodium thiosulfate aqueous solution for 2 min, and rinsed with water. Counterstaining was then applied with 0.1% nuclear fast red solution, followed by dehydration with gradient alcohol and mounting with neutral gum. The slides were then sent for microscopic examination. Under high magnification, the calcium deposition site appeared as black particles; the red dots were cell nuclei.

Statistical analysis

The measured data are presented as the mean±standard deviation (χ̄±s) and were analysed by SPSS16.0 statistical software. Levene’s test was used for a homogeneity test of variance (test level 0.10). A one-way ANOVA analysis was used among different age groups of the same type with equal variance. The non-parametric rank and test were used to compare the groups with different variances. An unpaired Student’s t-test was used to compare two groups with the same ages from different rat models; p<0.05 was considered statistically significant, and p<0.01 was considered highly statistically significant.

![Fig. 1. Dynamic changes of SBP in rats. All the data were presented as the χ̄±s. ▲: p<0.01, compared to the previous SHR group; #: p<0.01, compared to the age-matched WKY group.](image-url)
Results

The animal survival and growth conditions

A total of 100 rats were used in this experiment, and 4 of them died during the experimental procedure: 1 in 72 w WKY group, 1 in 48 w SHR group, and 2 in 72 w SHR group. The other 96 mice all completed the trials. The rats in both the WKY and SHR groups showed different extents of body size decrease, reduced hair gloss with yellowish colour, fluffy and withered hairs, hair loss, curved spine, slow response and other ageing characteristics. These features appeared gradually after 48 w, and became most obvious at 72 w. Compared to WKY rats, SHR showed more significant ageing phenotypes.

Fig. 2. Dynamic changes of morphology in the thoracic aortas of rats. A. HE staining. B. EVG staining. Scale bars: A, 20 μm; B, 500 μm and 20 μm.
Systolic blood pressure (SBP)

As shown in Fig. 1, for WKY rats, SBP did not show significant changes during ageing (4-72 w). However, in the SHR group, SBP increased with age: SBP was within the normal range at 4 w; then, it increased significantly at 12 w (182.57±8.11 mmHg) and reached the peak at 24 w (209.47±9.33 mmHg); after that, SBP was maintained at the peak level (24 w) for 48 w and 72 w. Except for the 4 w group (p>0.05), the other age groups of SHR all showed significantly higher SBP than that of the age-matched WKY rats (p<0.01).

Aortic morphological changes

As shown in Fig. 2A, the aortic morphology was normal for juvenile and adolescent stages (4 w and 12 w); the fusiform tunica media cells were arranged in concentric circles with regular shapes. However, with ageing, the WKY 72 w group showed the following ageing-related remodelling features: tunica media thickening, increased cell layers and cell size, cell morphological changes, and irregular cell arrangement, with some cells growing perpendicular to the intima. Compared to SHR, WKY rats showed more tightly arranged tunica media cells. SHR exhibited the above features since 24 w old, which was significantly earlier than WKY rats. Additionally, the degree of remodelling in SHR increased with age and was significantly worse than the age-matched WKY rats.

For both WKY rats and SHRs, the ageing-related arterial thickening mainly occurred at the tunica media (Figs. 2B, 3). Compared with WKY rats, the aortic elastic lamina of SHR groups was straightened, and the media elastic lamina was twisted and fractured; also, the collagen fibre deposition increased and was distributed more along the two sides of the elastic lamina. Statistical data showed that the MT, LD and MA of both the WKY and SHR groups increased with age. The MT/LD ratio in WKY rats was increased only at 72 w (p<0.05), and the Collagen/MA ratio increased at 48 w and 72 w (p<0.05). For SHRs, the MT/LD ratio increased at 24 w (p<0.01), which was significantly earlier than the WKY rats (only increased at 72 w). Similarly, the Collagen/MA ratio of SHR significantly increased at 12 w (p<0.01), which was earlier than the WKY rats (increased since 48 w). Consistently, the indexes mentioned above were significantly higher in SHRs compared to WKY rats at all time points (12 w, 24 w and 48 w, p<0.01 for all). These results demonstrated that the above pathological changes related to the aorta remodelling occurred significantly earlier and were more severe in SHRs compared to WKY rats.

The dynamic changes of α-SMA expression in the aorta

α-SMA is a marker for vascular smooth muscle cell (VSMC) contraction phenotype. As shown in Fig. 4A, the major cell type in the reconstructed thickened aortic media is VSMCs. The immunofluorescence intensity of α-SMA in the aorta gradually decreased with ageing in both groups. This α-SMA reduction occurred earlier in the SHR group (48 w) and was more evident compared to the age-matched WKY rats at 48 w and 72 w; this finding was consistent with the changes in α-SMA protein levels examined by western blotting (Fig. 4B).
Ageing-related aorta remodelling and calcification in SHR

Fig. 4. Dynamic changes of α-SMA expression in the thoracic aortas of rats. A. Immunofluorescence staining of α-SMA (red). DAPI was used for nuclear counterstaining (blue). B. Western blot of α-SMA in the thoracic aorta. The SHR aortas exhibited reduced α-SMA expression levels compared with WKY aortas. Data are normalized to β-actin and presented as the χ̄±s. *: p<0.01, compared to the previous WKY group; ▲: p<0.01, compared to the previous SHR group; #: p<0.05, compared to the previous SHR group; #: p<0.01, compared to the age-matched WKY group; +: p<0.05, compared to the age-matched WKY group. Scale bars: 50 μm.

Fig. 5. Dynamic changes of ageing in the thoracic aortas of rats. SA-β-Gal staining, positive staining is indigo blue, indicating senescent cells. All the values are presented as the χ̄±s. *: p<0.01, compared to the previous WKY group; ▲: p<0.01, compared to the previous SHR group; #: p<0.01, compared to the age-matched WKY group. Scale bars: 50 μm.
As shown in Fig. 4B, the expression of α-SMA protein in WKY rats was decreased only at 72 w (p<0.01), while SHR displayed a lower level of α-SMA protein at 48 w (p<0.01), which was earlier than WKY rats and further decreased at 72 w (p<0.05). Compared to age-matched WKY rats, the expression of α-SMA protein in SHR rats was significantly lower at 48 w (p<0.01) and 72 w (p<0.05). These results suggested that the aortic contractile VSMCs gradually decreased with ageing, and the reduction was more significant in SHRs at 48 w and 72 w old.

Ageing-related changes in aorta senescent cells

As shown in Fig. 5, in WKY rats, only the 72 w group showed a small amount of indigo blue senescent cells. By contrast, in SHRs, the 24 w group already showed sporadic senescent cells, which gradually increased with ageing, and the 72 w group showed significantly increased senescent cells (p<0.01 for 24 w-72 w groups compared to the age-matched WKY rats).

Ageing-related changes in aortic calcification

Calcium content: As shown in Fig. 6, the calcium content of the aortic wall in both WKY rats and SHRs gradually increased with age. In WKY rats, only the 72 w group showed a higher calcium content than the other WKY groups (p<0.05), while in SHRs, the 48 w group already began to show an increased aorta calcium content (p<0.05), and the 72 w group was more significant (p<0.01). The aorta calcium contents of 48 w and 72 w groups in SHRs were significantly higher than the age-matched WKY rats (p<0.05 and p<0.01).

The aortic media calcification was examined by von Kossa staining (Fig. 7); the black particles are the calcium deposition, which was mainly linearly deposited along the elastic fibres of the vessel tunica media and increased with age. Both WKY rats and SHRs showed small amounts of calcium deposition in the aorta at 4 w. WKY rats started to show a more abundant distribution at 72 w, while SHRs showed more calcium deposition at 48 w. The calcium deposition of SHRs in the 48 w and 72 w groups were significantly more than the age-matched WKY rats.
Ageing-related aorta remodelling and calcification in SHR

Discussion

To exclude the effects of external intervention, this study compared the dynamic changes of hypertensive rats with normotensive rats during the process of gradual blood pressure increases followed by a persistent high blood pressure level, which is concomitant with natural ageing. Based on the studies from Kieffer et al. (2000) and Kokubo et al. (2005), and using the age grouping method according to the rat behaviour and weight, we divided the rats into 5 groups. The blood pressure of SHRs was consistent with the literature (Okamoto and Aoki, 1963; Kokubo et al., 2005).

Ameer et al. (2014) compared the stiffness, nuclear density, medium thickness and cross-sectional areas of thoracic and abdominal aortic arteries in calcification model rats and SHRs and speculated that hypertension had a more balanced impact on large elastic arteries, and calcification had greater influence on the proximal aorta. Therefore, we selected the proximal aorta in this study.

VSMCs are the main component of vascular media. The excessive proliferation, abnormal migration and increased secretion are the major pathological changes in vascular ageing, causing and promoting ageing-related vascular remodelling (Yildiz, 2007; Lacolley et al., 2012). Ageing-related vascular calcification belongs to arterial media calcification (Demer and Tintut, 2008). Therefore, we chose the aortic media to perform the dynamic comparative study.

The dynamic changes of aorta remodelling, ageing and calcification in both WKY rats and SHR were investigated in this study. We found that the aorta remodelling, ageing and calcification occurred earlier and progressed more severely in SHRs compared to WKY rats. To the best of our knowledge, this is the first study to compare the dynamic changes between hypertensive rats and normotensive rats.

By studying the dynamic changes of aorta morphology in rats, we could compare the changes in the vascular wall structure caused by ageing and hypertension (Figs. 2, 3). The 72 w WKY group showed typical pathological changes of ageing-related vascular remodelling. SHRs started to show vascular morphological changes as early as 12 w, which was significantly earlier than WKY rats. Then, they showed gradual pathological changes of vascular remodelling that were similar to the aged WKY rats, but the changes were more severe than the age-matched WKY rats. Compared to WKY rats, the aortic remodelling of SHRs was featured with media thickening and increased collagen deposition; this finding was consistent with the results from Lin et al. (2015). Gauthier-Bastien et al. (2014) found that vascular media calcification was associated with significantly reduced alpha-SMA immunofluorescence signals, suggesting a decreased number of contraction-type vascular smooth muscle cells. Consistently, Zhang et al. (2010) found that in the in vitro culture system, the SHR-derived VSMCs exhibited a more obvious phenotypic switch from a contractile type to synthetic type compared to WKY-derived VSMCs, indicating the excessive VSMC phenotypic modulation under hypertension. In our study, we used an in vivo system and found consistent results. The aortic α-SMA immunofluorescence staining (Fig. 4A) and western blotting (Fig. 4B) showed that the major increased cell type in reconstructed aorta media was VSMCs, and the contraction type VSMC decreased with age, indicating that the VSMCs were transformed to a synthetic/proliferative type. Furthermore, the reduction of contractile VSMCs at 48 w and 72 w SHRs was more significant than the age-matched WKY rats.

SHR is the animal model in which the pathogenesis is close to human hypertension. With ageing, the appearance of aortic senescent cells in SHR (24 w old) was earlier than WKY rats (72 w old), and the number of senescent cells in SHRs was significantly higher than the age-matched WKY rats. Therefore, the arterial ageing of SHR was accelerated and more severe than in WKY rats (Fig. 5).

Ageing-related vascular calcification is the increase of calcium and phosphorus in the arterial wall with ageing (Demer and Tintut, 2008; Zhu et al., 2012). Our results showed that the calcium content of the arterial walls of WKY rats was only increased in the 72 w group (20% higher than 48 w group), while for SHRs, it was significantly increased at 48 w (22%) and further increased by 29% in the 72 w group compared to that in the 48 w group. The calcium contents of 48 w and 72 w SHR groups were significantly higher than those in the age-matched WKY rats (Fig. 6). The von Kossa staining (Fig. 7) showed that SHR exhibited more obvious calcium deposition with ageing compared to WKY rats. Moreover, by 72 w, the calcification of SHR was significantly more severe than the age-matched WKY rats. In this study, we used quantitative and qualitative methods to measure (Figs. 6, 7) the increase in calcium deposition with ageing and found the calcification of SHR thoracic aorta occurred earlier and progressed more severely than in WKY rats, demonstrating that both ageing-related vascular remodelling and calcification were accelerated and augmented in SHR aortas. These results support the findings from Fleckenstein et al. (1990), which demonstrated that the calcium content and calcification of SHR aortas were significantly higher than in WKY rats. In fact, Kanemaru et al. (2008) found that calcification was more likely to occur in the SHR-derived thoracic aorta VSMCs in vitro compared to the VSMCs derived from WKY rats. Altogether, these results confirmed that the thoracic aortas of SHRs were more susceptible for calcification both in vivo and in vitro.

Vascular remodelling under hypertension mainly involves intima and media of the elastic arteries (Engelen et al., 2013; Lakatta, 2013; Harvey et al., 2015). VSMCs are the main cell type in the media, continuously exposed to the mechanical signals and biochemical components generated in the artery. They are involved in all the physiological functions and the
pathological changes place in the artery. Due to their contractile tonus, VSMCs of resistance vessels participate in the regulation of blood pressure. VSMCs of conduit arteries also respond to a hypertension-induced increase in wall stress by up-regulating cell protein synthesis and extracellular matrix secretion, as well as phenotypic transformation and inducing vascular calcification (Lacolley et al., 2012). The effect of sustained hypertension on VSMCs was independent of age (Bruno et al., 2017). Our results confirmed that vascular ageing and calcification were accelerated and augmented in the aortas of SHRs; this finding was possibly related to the vascular remodelling mediated by VSMCs.

In summary, the physiological vascular ageing and hypertension superimposed on each other, interacting as reciprocal causations (Cunha et al., 2015), thus increasing the abnormalities of vascular structure and function. We found that the vascular remodelling, ageing and calcification of SHRs appeared earlier and progressed more severely than in WKY rats, demonstrating that both ageing-related vascular remodelling and calcification were accelerated and augmented in the aortas of SHRs, of which the underlying mechanisms remain to be investigated in the future.

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Conflict of interest. The authors declare that they have no conflict of interest.

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