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## Increased apoptosis in the heart of genetic hypertension, associated with increased fibroblasts

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### Abstract

**Objective:** The present studies were undertaken to identify apoptosis in cardiomyocytes of genetic hypertension and to study the relationship among apoptosis, aging and blood pressure, and the effect of angiotensin-converting enzyme (ACE) inhibitors on apoptosis. **Methods:** Apoptosis in the hearts of spontaneously hypertensive rats (SHR) was identified by electron microscopy (EM) and DNA laddering, and quantified from age 3 weeks to 64 weeks in comparison with normotensive rats (WKY). Fibroblasts and protein products of *Bcl-2* and *Bax* were measured by quantitative immunohistochemistry. SHR were treated with ramipril, an ACE inhibitor. **Results:** The results showed that: (1) ultrastructural characteristics of apoptosis were observed in cardiomyocytes of SHR, with shrinkage of the cell and condensation of the cytoplasm and chromatin. A DNA ladder was shown; (2) a significant increase in apoptosis in SHR began as early as age 4 weeks and reached a plateau at 16 weeks and maintained at high levels up to 64 weeks. Blood pressure (BP) in SHR started to increase significantly at age 5 weeks; (3) fibroblasts were significantly increased in the heart of SHR; (4) the ratio of *Bcl-2*/*Bax* was significantly reduced in SHR; and (6) ramipril effectively reduced apoptosis and fibroblasts, and increased the ratio of *Bcl-2*/*Bax*. **Conclusion:** Apoptosis occurs in the cardiomyocytes of genetic hypertension although fibroblasts are increased, and a significant, age-dependent increase in apoptosis is observed. The increase in apoptosis occurs before the difference in blood pressure is detectable. The ACE inhibitor ramipril may be useful for prevention of apoptosis in the heart. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Angiotensin; Apoptosis; Extracellular matrix; Fibrosis; Hypertension; Myocytes

### 1. Introduction

Apoptosis or program cell death is a gene-controlled cell suicide [1,2]. The role of apoptosis in diverse physiological processes and in a variety of pathological conditions has received considerable attention in recent years [1–3]. Apoptosis in heart and vasculature has recently been recognized.

Recently, some investigators have suggested that apoptosis may play a key role in heart disease, particularly heart failure [4]. The fundamental issue in heart failure is what causes the progressive loss of cardiomyocytes in a chronic process. As a death process induced by internal signalling triggered by imbalance in the biological conditions, apoptosis could be the cause for the loss of cardiomyocytes.

There are a variety of pathological conditions that eventually lead to heart failure. Hypertension is one of the very important conditions. Hypertension can result in diastolic and systolic heart failure. However, how this clinical setting eventually results in heart failure is unknown. We hypothesise that apoptosis could be involved in the mechanism that causes the progressive loss of cardiomyocytes in the failing heart of hypertension.

The most characteristic features of apoptosis are the morphological changes recognisable by electron microscopy (EM) and the internucleosomal cleavage of genomic DNA into fragments that are discrete multiples of 185–200 bp that produce a typical DNA ladder after agarose gel electrophoresis (DNA laddering) [1,2]. Ultrastructural characteristics of apoptosis, e.g. condensation and margination of chromatin and shrinkage of the cell, which can only be clearly seen under EM, have been demonstrated in

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certain cells, mostly tumor cells. However, the ultrastructural morphology of apoptosis in cardiomyocytes of a hypertensive heart has not been reported. A recent study on infarct hearts with EM [5] has shown that the previous claimed 'apoptotic' myocytes in the infarct area of hearts may be oncotic myocytes with DNA fragmentation. That study highlights the importance of using EM for studies on apoptosis [6]. Since the apoptotic morphology remains the gold standard for identification of apoptosis, it is necessary to investigate this issue in the cardiomyocytes of a hypertensive heart.

Angiotensin-converting enzyme (ACE) inhibitors have been routinely used to treat heart failure and hypertension. Prevention of cardiovascular remodelling has been proposed as the key mechanism of the efficacy of these drugs [7]. However, the precise mechanism of how these drugs prevent cardiovascular remodelling is not very clear. It is known that apoptosis plays an important role in tissue remodelling and could also be involved in cardiovascular remodelling in pathological conditions. We hypothesise that prevention of apoptosis could be one of the mechanisms for the efficacy of ACE inhibitors. Ramipril, an ACE inhibitor, has a long-acting effect, can be used once daily and is welcomed by clinicians and patients. Whether ramipril has an effect on apoptosis is unknown.

In the present studies, we used spontaneous hypertensive rat (SHR) as a model (a genetic hypertensive model) to study the following issues: (1) Does apoptosis occur in the genetic hypertensive heart? (2) If yes, how is it related to the age and the development of hypertension? (3) Can the rate of apoptosis be changed by the ACE inhibitor ramipril? We use the spontaneously hypertensive model as an approach to study the issue of apoptosis in hypertension, because over 90% of patients with hypertension have spontaneous hypertension (or essential hypertension). These studies may also provide necessary information for our future studies on other models of hypertension.

## 2. Methods

### 2.1. Animals and reagents

Rats (72 male SHR and 60 WKY) were obtained from the animal house of Austin Hospital, Heidelberg, Australia. Ethic approval for using these animals has been granted by Animal Ethic Committee of Austin Hospital. BP was measured by the indirect tail-cuff technique (WandW recorder, model 8005) in preheated, lightly restrained rats. Hearts were obtained from SHR and WKY at different ages (age 3, 4, 6, 8, 16, 32 and 64 weeks). The hearts were isolated, put into liquid nitrogen immediately and stored at  $-70^{\circ}\text{C}$ . Ramipril, a gift from Hoechst Marion Roussel Pharmaceutical, was used to treat SHR, with untreated SHR and WKY as controls. Young SHR ( $n=6$ ) were treated with ramipril (1 mg/kg, once daily), and the

control SHR ( $n=6$ ) and WKY ( $n=6$ ) with distilled water, from 3 to 10 weeks of age. The other reagents were from Sigma Chemical (St Louis, MO) unless otherwise stated.

### 2.2. Transmission electron microscopy (EM)

Preparation of cardiac tissues of SHR or WKY was performed as previously described [8]. Briefly, portions of cardiac tissues from SHR or WKY were fixed in 2.5% glutaraldehyde in 0.1 M PBS at  $4^{\circ}\text{C}$  overnight. The fixed tissues were washed with distilled water, dehydrated through graded acetone, and embedded in Araldite-Epon resin (CIBI-GEIGY, Lane Cove, NSW, Australia). Thin sections were cut and stained with saturated uranyl nitrate and Reynolds lead citrate, and viewed in a JEOL-1200 EX electron microscope (JEOL, Tokyo, Japan).

### 2.3. DNA laddering

DNA extraction and electrophoresis of  $\alpha^{32}\text{PdCTP}$ -labelled DNA fragments (DNA laddering) was performed as previously described [8]. A 10- $\mu\text{l}$   $\alpha^{32}\text{PdCTP}$  labelled (see below) DNA sample (about 100 ng) was precipitated for 2 h in  $-20^{\circ}\text{C}$  ethanol containing 0.3 M sodium acetate and 0.01 M  $\text{MgCl}_2$  (final concentration) and centrifuged for 30 min,  $4^{\circ}\text{C}$ , at 12 000 g. The precipitated, labelled DNA was washed twice with 70% cold ethanol and unincorporated  $\alpha^{32}\text{PdCTP}$  removed. The labelled DNA was resuspended in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA), mixed with 5 ml of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% ficoll) and electrophoresed for 3 h at 5.0 v/cm in 1.0% agarose gel using 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA) as running buffer. The gel was removed and dried in a Gel-Drying Frame (Kem-En-Tec, Haraldsgade, Denmark) following gel equilibration with 3% of glycerol for 1 h. Dried gel was sealed in plastic wrap and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) with an intensifying screen for 2–3 h at  $-70^{\circ}\text{C}$ .

### 2.4. Saturation labelling assay

Saturation labeling of the 3' end of apoptotic DNA fragmentations from the cardiac tissues of SHR and WKY was performed as previously described [8]. A 20- $\mu\text{l}$  reaction mixture containing 4  $\mu\text{l}$  of 5X buffer (Boehringer-Mannheim, Germany), 2  $\mu\text{l}$  of 2.5 mM  $\text{CoCl}_2$ , 1000 ng DNA, and increasing concentrations of ddCTP (Pharmacia, Uppsala, Sweden) and  $\alpha^{32}\text{PdCTP}$  (Bresatec). The ratio of ddCTP: $\alpha^{32}\text{PdCTP}$  was 4.7:1 per reaction. (The increasing concentrations in pmol were 1.93:0.41, 3.85:0.82, 7.76:1.65, 15.5:3.3, 31.0:6.6, and 62.0:13.2 each in triplicate). After taking 2  $\mu\text{l}$  as background, 20 U TdT (Pharmacia, Uppsala, Sweden) were added. The reaction was proceeded for 60 min at  $37^{\circ}\text{C}$ , and stopped by adding 2  $\mu\text{l}$  of 0.5 M EDTA. Detection of the  $\alpha^{32}\text{PdCTP}$

incorporated into 3'-ends of DNA strand breaks was performed as follows: 2  $\mu$ l labeling sample was added to 48  $\mu$ l of 0.5 mg/ml calf thymus DNA as carrier. A 5- $\mu$ l aliquot of the mixture was spotted onto a glass-fibre filter (Whatman 2.5 cm GF/C) for assessing total radioactivity. The labelled DNA was precipitated by 1.0 ml 10% (V/W) trichloroacetic acid (TCA) for 10–15 min on ice. The unincorporated  $\alpha^{32}$ PdCTP was eluted through a second glass-fibre filter by washing four times with 3 ml 10% TCA, and three times with 3 ml 100% ethanol. The filter was dried and placed into a vial. The amount of incorporated  $\alpha^{32}$ PdCTP was detected by counting for 5 min in a 300 CD liquid scintillation counter (Packard Instrument). All experiments were repeated at least three separate times and in triplicate for each sample.

### 2.5. Calculations

The details of calculation have been described previously [8]. The  $L_{\max}$  of the saturation labeling assay was obtained as following:

$$B/F = (L_{\max} - B)/K_m$$

(the Scatchard equation, see Ref. [9])

$$L_{\max} = (K_m \cdot B)/F + B$$

$L_{\max}$  = maximal labeling or incorporation (pmol incorporated  $\alpha^{32}$ PdCTP/mg DNA)

$B$  = detected incorporated  $\alpha^{32}$ PdCTP (pmol incorporated  $\alpha^{32}$ PdCTP/mg DNA)

$K_m$  = kinetic parameter =  $-1/\text{slope}$  (see Fig. 2B)

$F$  = amount of  $\alpha^{32}$ PdCTP added in the reaction (pmol)

In order to obtain the  $L_{\max}$ , there are at least six increasing concentrations of  $\alpha^{32}$ PdCTP needed for a DNA sample. The data were analyzed by Scatchard plot through the 'simple curve fit' with the Microsoft version 5.1 (Microsoft, USA) in a Macintosh computer. The data are shown as  $L_{\max}$  in the application of the saturation labeling assay in quantification of apoptosis.

### 2.6. Quantification of Bcl-2, Bax and fibroblasts

The quantity of Bcl-2, Bax and fibroblasts were determined by a modified objectively quantitative immunohistochemistry, originally developed in our laboratory [10]. The hearts were obtained at age 11 weeks. The cardiac tissues and coronary arteries were cut into tissue sections of 14  $\mu$ m. The sections were fixed/dehydrated in acetone at room temperature for 10 min, dried, and rehydrated in 0.05 M TrisCl pH 7.4 for 5 min before an incubation with 20% swine serum for 20 min in order to decrease nonspecific binding. The sections were then incubated with antibodies to Bcl-2, Bax, or fibroblasts with a specific monoclonal antibody to the beta-subunit of prolyl-4-hydroxylase of fibroblasts, which is a specific cell component

of fibroblasts (Dako Australia, Botany, NSW; 250  $\mu$ g/ml diluted in 0.05 M TrisCl pH 7.4 containing 1% BSA). Peptide neutralization was used as our negative control. Following three 10-min rinses to remove the excess antibody, a 5-min incubation with a biotinylated anti-rabbit antibody at 1:200 dilution was used to incubate the tissue sections. Once more, three rinses in 0.05 M TrisCl pH 7.4 for 10 min were performed to remove excess biotinylated antibodies. In the following 5-min incubation, a streptavidin molecule was used to conjugate to alkaline phosphatase at a 1:200 dilution. Subsequently, three 10-min rinses in 0.05 M TrisCl pH 7.4 in a gentle rocking manner was performed. The tissue sections were then equilibrated in 100 mM TrisCl, pH 9.5 containing 100 mM NaCl for 2 min. To provide chemiluminescence, the sections were incubated with CDP-Star substrate (diluted at 1:200 in 100 mM TrisCl, pH 9.5, 100 mM NaCl), for five min. The substrate included 10 mM Levamisole to inhibit endogenous alkaline phosphatases. The sections were then dried in an oven at 20°C and then exposed for 10 min under Mamoray MR5 X-ray film. The following images were analyzed using a microscopy computer imaging devise (MCID) [10].

### 2.7. Statistical analysis

Results are expressed as mean  $\pm$  S.E. Student's test was used for two group comparison and a two-way analysis of variance for multi-group comparisons and a three-way analysis of variance for difference among species, treatment groups and ages (experiments). Statistical significance was taken at a level of  $P < 0.05$ .

## 3. Results

### 3.1. Detection of apoptosis in cardiomyocytes of SHR

The morphologic characteristics of apoptosis were clearly observed in cardiomyocytes of SHR under EM including shrinkage of nuclear volume, margination and condensation of chromatin (Fig. 1b, c). A normal cardiomyocyte obtained from WKY was used as control. The nucleus (N) in the normal cell (Fig. 1a) showed even distribution of chromatin and a normal nucleolus (n). An apoptotic cell from SHR illustrates early ultrastructural changes of apoptosis (Fig. 1b). The nucleus (N) showed shrinkage characterized by the appearance of a clear gap around the nucleus and contained small marginated electron dense chromatin masses (arrow). Clumped ill-defined nuclear chromatin was also present (arrowhead). Another cell (Fig. 1c) manifests further advanced apoptosis compared with the cardiomyocyte in Fig. 1b. The nucleus (N) showed deep condensation that was revealed by the appearance of a large gap around the nucleus and invaginations of the nucleolemma, and contained large sharply

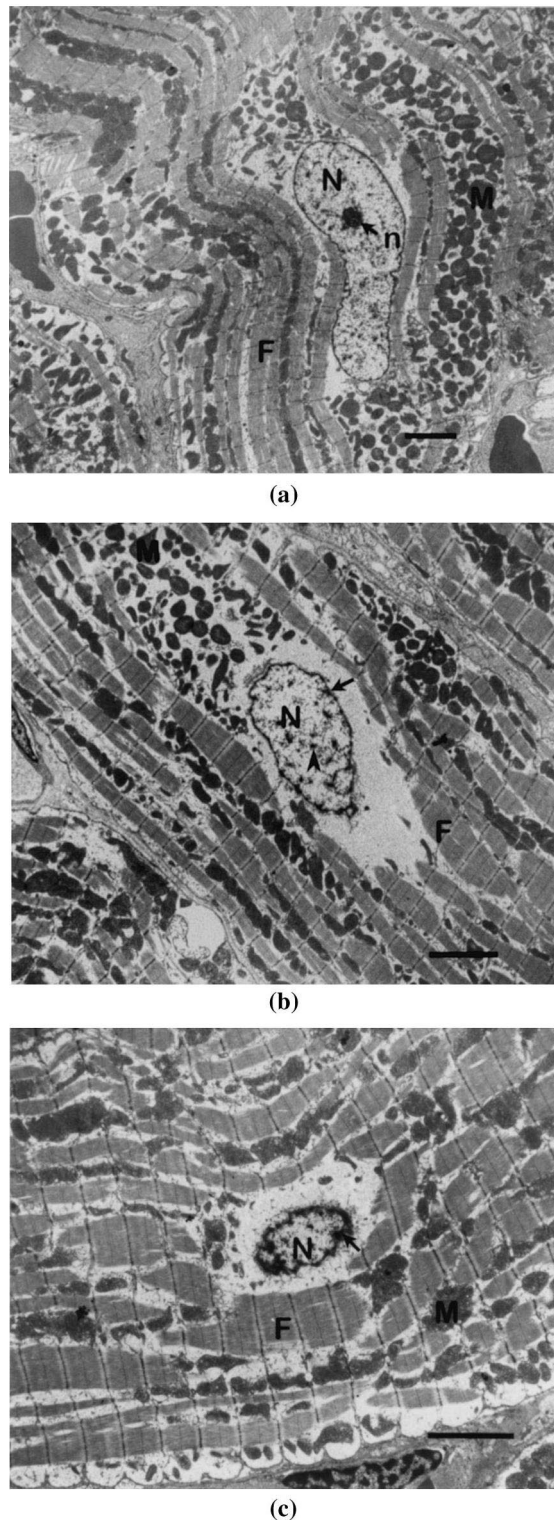


Fig. 1. Transmission electron micrographs of cardiomyocytes from the left ventricle of WKY (A) and SHR (1b and 1c) at age 16 weeks. A normal cardiomyocyte from WKY was used as control (a). Early ultrastructural changes of apoptosis in cardiomyocyte of SHR are shown in (b). A further advanced apoptotic cardiomyocyte of SHR is shown in (c). (See text for details). N: nucleus, n: nucleolus, arrow (Fig. 1b and c): small marginated electron dense chromatin masses, arrowhead (Fig. 1b): clumped ill-defined nuclear chromatin, M: mitochondria, F: myofibrils. Bar: 4  $\mu\text{m}$ .

marginated electron dense chromatin masses that abutted the nuclear envelope as well as large clumped ill-defined nuclear chromatin. These results identify that the cardiomyocytes of SHR undergo apoptosis. Apoptosis in SHR was also shown by the electrophoresis of DNA ladder pattern that showed a typical nucleosome ladder in cardiac tissue obtained from SHR (Fig. 2, lane 1,3).

### 3.2. Quantification of DNA fragments in the cardiac tissues of SHR

DNA fragments of the cardiac tissues in SHR and WKY were quantitated at age 3, 4, 6, 8, 16, 32 and 64 weeks. The results showed that there was a significant age-dependent increase in apoptosis in SHR, and the labeling of DNA breaks in SHR started to increase significantly at age 4 weeks ( $L_{\text{max}}$ :  $12.34 \pm 0.94$  pmol/mg DNA in SHR vs.  $9.97 \pm 0.78$  in WKY,  $P < 0.05$ ) and reached a plateau at 16 weeks ( $37.90 \pm 2.84$  pmol/mg DNA) (Fig. 3a). The values were maintained at high levels up to 64 weeks ( $39.35 \pm 2.5$  in SHR vs.  $11.44 \pm 1.78$  in WKY,  $n = 6$ ,  $P < 0.01$ ). When the DNA from WKY was tested, there was no significant increase in the labeling of DNA breaks with increasing age, although the trend showed a slight gradual increase in apoptosis from age 3 weeks to 64 weeks (the values of  $L_{\text{max}}$  were  $9.90 \pm 0.81$  pmol/mg DNA at 3 weeks vs.  $11.44 \pm 1.78$  at 64 weeks,  $n = 6$ ,  $P > 0.1$ ) (Fig. 3a). Meanwhile, the blood pressure of these SHR started to increase significantly at age 5 weeks, and reached a plateau at 16 weeks (Fig. 3b). Interestingly, the increase in DNA fragmentation in SHR was earlier than the occurrence of the significant increase in BP.

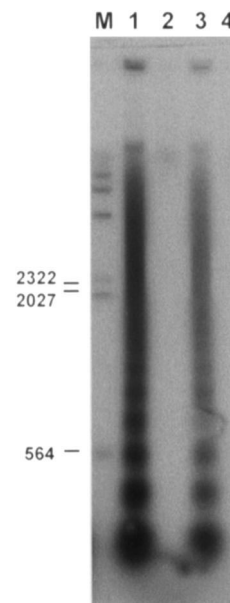


Fig. 2. Autoradiographic analysis of DNA extracted from cardiac tissues of SHR (lane 1,3) or WKY (lane 2,4) at age 16 weeks. Lane 1 and 3, 1 mg DNA; lane 2 and 4, 0.5 mg DNA; lane M, molecular markers.

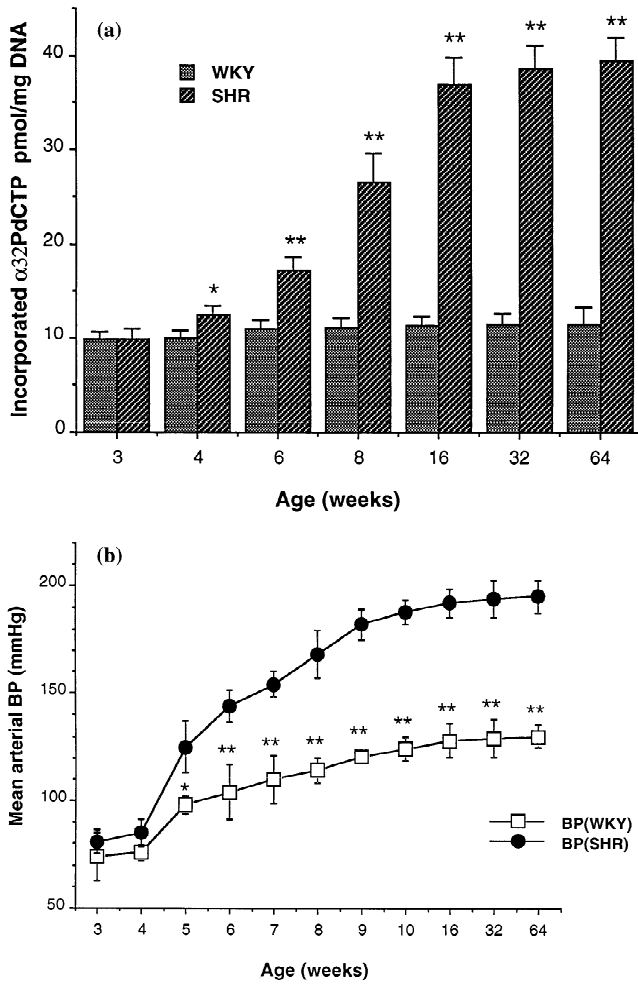


Fig. 3. (a) DNA fragmentation at different ages of SHR or WKY. DNA was isolated and quantitated from cardiac tissues of SHR (■) or WKY (□) at age 3, 4, 6, 8, 16, 32 and 64 weeks. A significant increase in DNA fragmentation occurred from age 4 weeks in SHR, reached a plateau at approximately 16 weeks, and maintained at high levels up to 64 weeks. Values are  $L_{max}$  (see Methods). (b) Mean blood pressure of WKY (□) and SHR (●) measured at age 3 to 64 weeks ( $n=6$ ). A significant increase in blood pressure occurred from age 5 weeks, reached a plateau at approximately 16 weeks, and was maintained at high levels up to 64 weeks. Values represent means  $\pm$  S.E., \* $P<0.05$ , \*\* $P<0.01$ ,  $n=6$ .

### 3.3. Fibroblasts in the hearts

The results showed that the quantity of fibroblasts was significantly increased in the hearts of SHR ( $P<0.01$ ,  $n=6$ ), compared to those of WKY (Fig. 4). Treatment of SHR with ramipril significantly reduced the quantity of fibroblasts compared with untreated SHR ( $P<0.01$ ,  $n=6$ ) (Fig. 4).

### 3.4. Bcl-2 and Bax in the hearts

Bcl-2 proteins were significantly lower in SHR than in WKY ( $P<0.01$ ,  $n=6$ , Fig. 5). In contrast, Bax proteins were significantly higher in SHR than in WKY ( $P<0.01$ ,

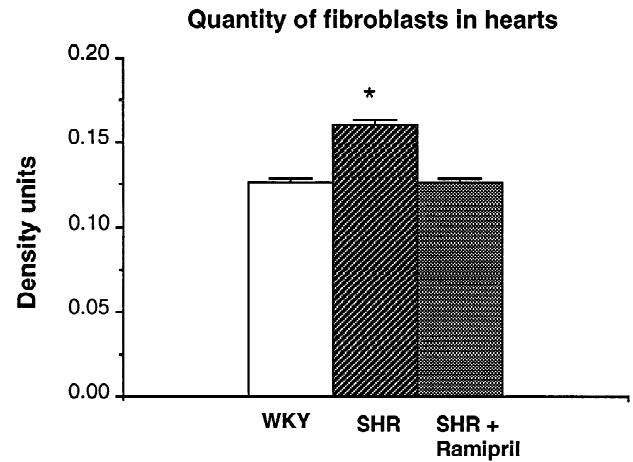


Fig. 4. Graph showing the difference between SHR and WKY in the amount of fibroblasts in hearts and the effect of ramipril on fibroblasts of the hearts of SHR. Fibroblasts were significantly increased in the hearts of SHR. A group of SHR were treated with ramipril (1 mg/kg, once daily) and the untreated SHR and WKY were treated with distilled water from age 3 to 10 weeks. Treatment of SHR with ramipril was associated with a significant reduction in the quantity of fibroblasts. Density units: MCID unit/pixel. Values represent means  $\pm$  S.E., \*\* $P<0.01$ ,  $n=6$ .

$n=6$ , Fig. 5). Ramipril increased the quantity of Bcl-2 proteins and reduced that of Bax ( $P<0.01$ ,  $n=6$ , Fig. 5).

### 3.5. Effect of ACE inhibitor on apoptosis

The DNA fragmentation of SHR treated with ramipril was significantly reduced ( $L_{max}$ ,  $12.94 \pm 2.30$  pmol/mg DNA) compared to untreated SHR ( $L_{max}$ ,  $27.20 \pm 2.30$  pmol/mg DNA) ( $P<0.01$ ) (Fig. 6), while there was no significant difference in the DNA fragmentation between the SHR treated with ramipril and WKY control ( $L_{max}$ ,

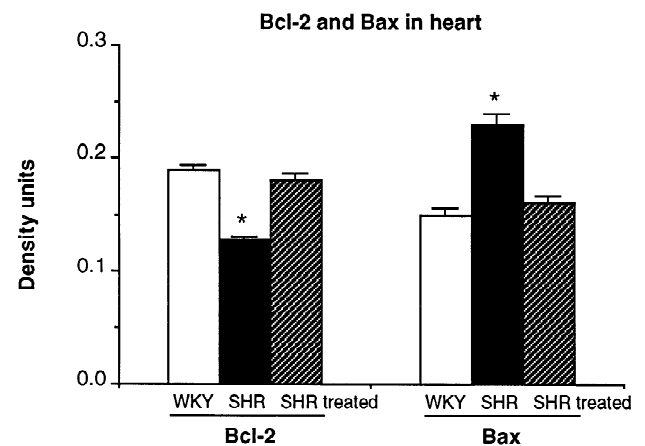


Fig. 5. Graph showing the difference between SHR and WKY in the amount of Bcl-2 and Bax in cardiac tissues and the effect of ramipril on these regulatory proteins. Bcl-2 was significantly increased in SHR, but Bax was significantly reduced in SHR. Treatment of SHR with ramipril (1 mg/kg, once daily) resulted in a significant increase in Bcl-2 and a significant reduction in Bax. Density units: MCID unit/pixel. Values represent mean  $\pm$  S.E., \* $P<0.01$ ,  $n=6$ .

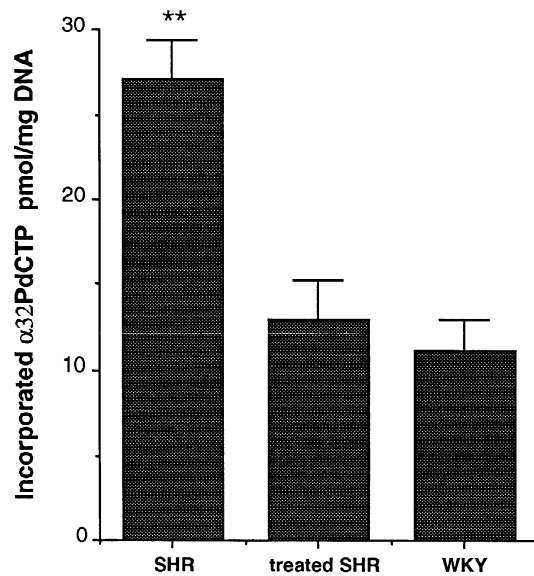


Fig. 6. Effect of ramipril on DNA fragmentation of the hearts of SHR. A group of SHR were treated with ramipril (1 mg/kg, once daily) and the untreated SHR and WKY were treated with distilled water from age 3 to 10 weeks. Treatment of SHR with ramipril was associated with a significant reduction in DNA fragmentation. Values represent means  $\pm$  S.E., \*\* $P < 0.01$ ,  $n = 6$ .

11.80  $\pm$  1.0 pmol/mg DNA) ( $n = 6$ ,  $P > 0.1$ ) (Fig. 6). Meanwhile, the ratio of the heart:body weight as an index of cardiac hypertrophy was significantly different between untreated SHR and treated SHR ( $P < 0.05$ ), and no significant difference between WKY and SHR treated with ramipril ( $P > 0.10$ ). Results also displayed that there was no effect of the ramipril treatment on body weight ( $n = 6$ ,  $P > 0.20$ ) over the treatment period from 3 to 10 weeks of age. The untreated SHR showed a steady increase in BP, characteristic of the developmental phase of hypertension. At age 10 weeks, BP was significantly lower in the group treated with ramipril than in untreated SHR (the untreated, 188  $\pm$  7.0 mmHg; the ramipril-treated, 128  $\pm$  5.2 mmHg,  $n = 6$ ,  $P < 0.001$ ), and there was no significant difference between the ramipril-treated SHR and WKY (WKY, 124  $\pm$  5.6; the ramipril-treated SHR, 128  $\pm$  5.2 mmHg,  $P > 0.10$ ).

#### 4. Discussion

There are five main findings in the present studies: (1) ultrastructural characteristics of apoptosis in the cardiomyocyte of SHR were identified by EM and apoptosis in SHR was also shown by the biochemical method DNA ladder pattern; (2) an age-dependent significant increase in apoptosis occurred in SHR from age 4 weeks, reached a plateau at age 16 weeks and maintained at high levels up to 64 weeks; (3) the increase in apoptosis appeared earlier (age 4 weeks) than did the increase in blood pressure (5 weeks); (4) The quantity of fibroblasts was significantly

increased in the heart of SHR; (5) the ratio of Bcl-2/Bax was significantly reduced in SHR; and (6) ramipril effectively reduced apoptosis and fibroblasts, and increased the ratio of Bcl-2/Bax.

We have revealed a typical morphology of apoptosis in the cardiomyocytes of SHR. The results clearly demonstrate that apoptosis occurs in the cardiomyocytes of SHR. Since we have detected apoptosis in SHR by four different methods (electron microscopy, DNA laddering, quantification of DNA fragmentation, and quantification of apoptotic regulatory proteins), we feel confident that apoptosis does occur in genetic hypertension.

By quantification, the present studies demonstrate the time course for the development of apoptosis, hypertension and age. It indicates clearly that apoptosis is increased in genetic hypertension and is associated with aging. The studies also demonstrate that the increase in apoptosis occurs earlier (4 weeks) than does BP (5 weeks). There are two explanations for this phenomenon. One is the tail-cuff method for measurement of BP may not be sensitive enough to detect the subtle increase in BP in the early life of SHR in which an increase in apoptosis has been detected. The other explanation is that the triggering of apoptosis may not depend on BP but on other factors such as neurohumoral factors that also cause hypertension. Studies by other investigators have shown that angiotensin II (AII), a blood pressure producer, can cause apoptosis in cultured cardiomyocytes [11]. This direct evidence suggests that apoptosis in SHR might not, or at least not solely, depend on blood pressure.

One question is in which cell types did the increased apoptosis occur? The heart consists of many cell types. Besides cardiomyocytes, fibroblasts are another major group of cells in heart. It could be possible that the increased apoptosis is caused by an increase in apoptosis of fibroblasts. However, in the present studies the quantity of the fibroblasts was actually increased but not reduced in SHR. We believe that the increased apoptosis most likely occurs in cardiomyocytes, which has actually been observed in our EM results. However, the possibility of increased apoptosis in fibroblasts cannot be completely ruled out, because apoptosis and mitosis in fibroblasts may both increase but the balance between them may be in favour of mitosis in this case.

The progressive loss of cardiomyocytes in the heart may play a key role in pathogenesis of heart failure (including diastolic and systolic failure). It is well known that adult cardiomyocytes do not undergo mitosis. Therefore, the lost cardiomyocytes cannot be re-grown, but can be replaced by other cells such as fibroblasts (In fact, our results did show an increase in fibroblasts in SHR). Studies [12] have been reported that cardiomyocytes are gradually lost in aging rat heart, which is accompanied with a gradual increase in fibrous tissues, although the cardiomyocyte volume is increased (hypertrophy) in the aging heart. The morphologic changes result in elevated left ventricular

end-diastolic pressure and decreased  $dP/dt$  [12], which indicates that a significant impairment of ventricular function occurs with senescence. In our studies, there was a gradual slight increase in apoptosis with aging in WKY. Although the increase is not statistically significant it does show the trend (to 64 weeks only). We believe that a significant increase in apoptosis in the pathological conditions of hypertension speeds up the process to heart failure. Therefore, to reduce apoptosis in the heart could become a therapeutic target for future treatment of hypertension and heart failure.

Our present studies showed that the treatment of genetic hypertension with ramipril was associated with a reduction in apoptosis and a prevention of an increase in fibroblasts. Our results also showed that ramipril interfered a signal transduction pathway of apoptosis: the regulatory proteins Bcl-2 and Bax. The protein product of oncogene *Bcl-2* is anti-apoptotic and that of *Bax* is pro-apoptotic. Their activity appears to control the cell death pathway at a checkpoint between signals from the cell surface and activation of the effector proteases [13]. It is proposed that reduction of the ratio of Bcl-2/Bax activates apoptosis. Direct evidence that AII stimulates apoptosis in cultured cardiomyocyte has been reported [11]. Taken these together, it is likely that the mechanism by which ACE inhibitors improve heart failure may be at least in part due to prevention of apoptosis in cardiomyocytes, which prevents cardiac remodelling.

One argument is that reduction of hypertrophy of the heart may be achieved by stimulation of apoptosis with a pharmaceutical agent. We believe that may not be true in cardiomyocytes, because adult cardiomyocytes do not undergo mitosis and the hypertrophy is caused by enlargement of the cell volume. Stimulation of apoptosis can only reduce the number of the existing cardiomyocytes, which may do more harm than good because the progressive loss of cardiomyocytes is the fundamental problem that leads to heart failure.

In conclusion, the present studies demonstrate that apoptosis may be involved in the pathogenesis of genetic hypertension and heart failure. The inhibition of apoptosis in the heart may be one of the mechanisms of ACE inhibitors to prevent cardiac remodelling in hypertension and heart failure.

## Acknowledgements

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