

Doxazosin modifies Bcl-2 and Bax protein expression in the left ventricle of spontaneously hypertensive rats

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Background Increased apoptosis has recently been reported in the heart of spontaneously hypertensive rats (SHRs).

Objective To investigate the molecular basis of apoptosis in the left ventricle of SHRs in terms of the expression of Bcl-2 protein (which protects from apoptosis) and Bax protein (which acts as an apoptotic promoter). In addition, we analysed the involvement of α_1 -adrenergic receptors in the left ventricular apoptosis of SHRs.

Methods The study was performed in untreated SHRs ($n = 16$) and SHRs that were orally treated with doxazosin (10 mg/kg body weight per day, for 15 days), a selective α_1 -receptor blocker ($n = 16$). A group of Wistar-Kyoto (WKY) rats ($n = 16$) was used as the control.

Results The left ventricles of untreated SHRs showed a significant increase in Bcl-2 protein expression and a reduced presence of Bax protein. The ratio of Bcl-2:Bax in SHRs was higher than in WKY rats, suggesting an anti-apoptotic state. Paradoxically, both the number of apoptotic cardiac cells and the cleavage of an 85-kDa fragment of the poly (ADP-ribose) polymerase (PARP), a marker of caspase-3 activity, were higher in the left ventricle of SHRs than in WKY rats, suggesting an apoptotic situation. Bax promotes cell apoptosis when it is bound to Bcl-2. We then determined the abundance of Bax-Bcl-2 complexes in the left ventricle of the two groups of animals. Bax-Bcl-2 complexes were more abundant in SHRs than WKY rats. In a second set of

experiments, we analysed the role of α_1 -adrenergic blockade by doxazosin in the above-described mechanisms. Doxazosin treatment reduced the formation of Bax-Bcl-2 complexes in the left ventricle of SHRs, and this was accompanied by a decrease in the levels of 85-kDa PARP and a reduction in apoptotic left ventricular cells.

Conclusions The present work suggests that the presence of Bax-Bcl-2 complexes in the left ventricle could be a more reliable marker of the apoptotic state than the determination of the absolute expression of Bcl-2 and Bax proteins. Moreover, the inhibition of α_1 -adrenergic receptors by doxazosin decreased the abundance of Bax-Bcl-2 complexes and promoted a reduction of apoptosis in the left ventricle of SHRs. *J Hypertens* 2000; 18:307-315 © Lippincott Williams & Wilkins.

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Introduction

Apoptosis is a process that regulates cell mass and architecture in many tissues. Apoptosis, also known as programmed cell death, is tightly regulated by specific intracellular signalling pathways that ultimately induce cell destruction [1,2]. Although those pathways may differ in different tissues, they usually converge in common cell death mechanisms [3]. The Bcl-2 proto-oncogene family has been postulated as one of the critical mechanisms by which cell death is determined [4,5]. The Bcl-2 family members are divided into those that protect the cell from apoptosis, that is Bcl-2, and

those that induce apoptosis, that is Bax. Other authors have reported that cell viability may depend on the changes in the level of expression of Bcl-2 and Bax proteins [5,6]. Thus, the ratio of the apoptotic Bax protein to its anti-apoptotic homologue Bcl-2 seems to be a main determinant of relative resistance to stimuli that can initiate cell death [6]. Members of the Bcl-2 family share two homologous regions, BH1 and BH2, that allow them to interact [6]. There is now growing evidence to suggest that Bax protein can neutralize the action of Bcl-2 protein by forming heterodimers with Bcl-2 [6,7].

Apoptosis has been demonstrated to occur in the myocardium in a variety of pathological situations [8]. The number of apoptotic cardiac cells is increased in the myocardium of patients with end-stage heart failure and myocardial infarction [9,10]. Moreover, Hamet *et al.* [11] have demonstrated apoptosis in the heart of spontaneously hypertensive rats (SHRs); other authors [12,13] have shown that this takes place in the left hypertrophied ventricle of hypertensive genetic animal models.

However, how this occurs is not yet clear and further investigation into the molecular mechanisms of left ventricular apoptosis is required. A significant role for α_1 -adrenergic receptors in the development of left ventricular hypertrophy associated with hypertension has been suggested by several clinical and experimental observations [14,15]. However, the involvement of α_1 -receptors in apoptotic pathways in the hypertrophied left ventricle is largely unknown. Accordingly, we examined the effects of an α_1 -blocker, doxazosin, on left ventricular apoptosis in SHRs.

Material and methods

Experimental groups

The study was performed in 17-week-old male SHRs (Iffa-Credo, L'Abresle, France) and their normotensive genetic controls, Wistar-Kyoto (WKY) rats, which were divided into three experimental groups: 17-week-old SHRs ($n = 16$) who were treated orally with doxazosin (Pfizer Pharmaceuticals, New York, New York, USA), a selective α_1 -receptor blocker [16], for 2 weeks before being killed at 19 weeks; untreated 17-week-old SHRs ($n = 16$) who were maintained for 2 weeks and then killed at 19 weeks; and a group of 17-week-old WKY rats ($n = 16$) who were killed at 19 weeks. Doxazosin was dissolved in the drinking water and the concentration was adjusted for the daily water intake and body weight to obtain an average daily dose of 10 mg/kg body weight per day. All rats were housed in individual cages and were fed with standard rat chow.

The animals were manipulated following procedures approved by the Animal Research Committee of Fundación Jiménez Díaz. The mean arterial pressure (MAP) was measured by the standard tail-cuff method using a Pressure Computer System (Power Lab 400, AD Instruments, Casterhill, New South Wales, Australia).

Preparation of tissue samples

All animals were anaesthetized with sodium pentobarbital (200 mg/kg body weight) and killed at the age of 19 weeks. The heart was removed after the right atrium was incised to allow the drainage of blood. Eight of the animals from each group were used for immunohistochemical determinations. For this purpose, a cannula

was inserted into the abdominal aorta and connected to a perfusion pump. Perfusion was performed with 50 ml saline to wash out the blood, followed by 200 ml fixative solution containing 4% buffered paraformaldehyde at a perfusion pressure that was the last arterial pressure recorded in each animal. The left ventricle was isolated, postfixed for 4 h (4% paraformaldehyde in 0.1 mol/l phosphate buffered saline) at room temperature and embedded in paraffin.

The hearts of the remaining eight of the animals were processed for Western blotting. First, the cardiac weight was measured to determine the cardiac index, which was calculated by dividing the heart weight by the body weight of each animal.

The left ventricles were frozen in liquid nitrogen for Western blot determinations.

Western blot and immunoprecipitation

Western blots were used to measure Bcl-2 and Bax proteins. Briefly, Bcl-2 and Bax protein expression was analysed by pulverizing and solubilizing the left ventricle of the different groups of rats in Laemmli buffer containing 2-mercaptoethanol [17].

Proteins were separated on denaturing sodium dodecyl sulphate (SDS)-15% polyacrylamide gels (PAGE). Equal amounts of proteins (20 μ g/lane) estimated by bicinchoninic acid reagent (Pierce, Rockford, Illinois, USA) were loaded. To verify that equal amounts of protein had been loaded onto the gel, a parallel gel with identical samples was run and stained with Coomassie to compare the intensities of the protein bands. As described elsewhere [18,19], the separated proteins were then blotted into nitrocellulose (Immobilon-P, Millipore Corporation, Bedford, Massachusetts, USA). The blots were blocked overnight at 4°C with 5% nonfat dry milk in TBS-T (20 mmol/l Tris (hydroxymethyl) aminomethane (Tris-HCl), 137 mmol/l NaCl, 0.1% Tween 20). Western blot analysis was performed with polyclonal antibodies against Bax and Bcl-2 (Calbiochem, La Jolla, California, USA). The blots were incubated with the first antibody (1:1000) for 1 h at room temperature and, after extensive washing, with the second antibody (horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody) at a dilution of 1:1500 for a further 1 h. Specific Bcl-2 and Bax proteins were detected by enhanced chemoluminescence (ECL, Amersham Iberica, Madrid, Spain) and evaluated by densitometry (Molecular Dynamics, Sunnyvale, California, USA). Prestained protein markers were used for molecular mass determinations.

For immunoprecipitation, lysates (100 μ g total protein per sample) from the left ventricle of the different groups of rats were precleared by adding 2 μ g normal

rabbit IgG and 10 µl protein-A-Agarose (Affinity Research Products Ltd, Exeter, UK) for 30 min at 4°C. Precleared lysates were obtained by centrifugation at 2500 rpm for 5 min at 4°C. The supernatants were recovered and incubated in the presence of anti-Bcl-2 antibody (AC21, Santa Cruz Biotechnology Inc, Santa Cruz, California, USA) for 1 h at 4°C. Twenty micro-litres protein A-Agarose was then added to the supernatant, which was then incubated overnight at 4°C. Immunoprecipitates were collected by centrifugation at 2500 rpm for 10 min at 4°C. The pellet and 10 µl supernatant were resuspended in 20 µl Laemmli buffer and the proteins were separated in 15% SDS-PAGE. The amount of Bax protein that formed complexes with Bcl-2 was detected by immunoblotting using an anti-Bax antibody as already described for the Western blot analysis.

Immunohistochemical studies

Two sections of the left ventricle of each rat were independently processed for Bcl-2 and Bax detection using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [20]. In brief, paraffin blocks were sectioned at 4 µm on a standard rotary microtome (Microm HM 325, Microm GmbH, Walldorf, Germany). The sections were stained with haematoxylin and eosin using an automated staining system. The sections were submitted to antigen retrieval for 2 min in a pressure cooker [21] and subsequently incubated with polyclonal antibodies against Bax and Bcl-2 (Calbiochem, La Jolla, California, USA) at a dilution of 1:100 overnight at 4°C. A mouse anti-rabbit IgG antibody (Dako Corporation, Carpinteria, California, USA) was applied for 30 min at room temperature and subsequently incubated with rabbit antimouse IgG (Dako Corporation, USA) and preformed APAAP immune complexes for another 30 min. The sections were washed in phosphate buffered saline containing 1% Tween-20 between each incubation. The colour was finally developed with Fast Red substrate (Dako Corporation, USA). After washing in deionized water, the sections were briefly counterstained with haematoxylin and coverslipped.

Flow cytometry analysis and caspase-3 protease activity

For the flow cytometry study, the cellular suspension was obtained according to the method previously described by Vindelov and Christensen [22]. The samples were placed in phosphate-buffered saline and cut with scissors. After centrifugation at 1500 rpm for 5 min, the cells were treated with ribonuclease A (10 µg/ml, Sigma, St Louis, Missouri, USA) and the DNA was stained with propidium iodide (100 µg/ml) in 0.05% Nonidet P-40 (Fluka Chemie AG, Busch, Switzerland). Analysis was performed in an EPICS-XL flow cytometer (Coulter, Hialeah, Florida, USA) as described [23]. The analysis of apoptotic cells was carried out using the Mcycle program. The apoptotic popula-

tion showed a low stainability, resulting in a quantifiable region below the G₀/G₁ peak. Furthermore, apoptotic cells showed lower side forward scatter and higher side scatter values than viable cells, reflecting their smaller size and different consistency. A minimum of 5000 cells were analysed for each sample.

During apoptosis, caspase-3 cleaves the 116-kDa substrate poly (ADP-ribose) polymerase (PARP) into a stable 85-kDa fragment containing the carboxy terminal and a 25-kDa fragment [24,25]. We then determined the level of the 85-kDa form as an index of apoptosis. The 85-kDa form was detected by immunoblotting as described above using an anti-PARP polyclonal antibody (Santa Cruz Biotechnology Inc). The proteins obtained from the left ventricle of the different rats had previously been separated in 12.5 % SDS-PAGE. The anti-PARP antibody (1:500) corresponded to amino acids 764–1014 mapping at the carboxy terminus of PARP.

Statistical analysis

Results are expressed as means ± SEM. Unless otherwise stated, each value corresponds to a minimum of six different rats from each experimental group. To determine the statistical significance of our results, we have performed an ANOVA with Bonferroni's correction for multiple comparisons or a Student's *t* test (paired or unpaired). A *P* value < 0.05 was considered statistically significant.

Results

Blood pressure and left ventricular hypertrophy parameters

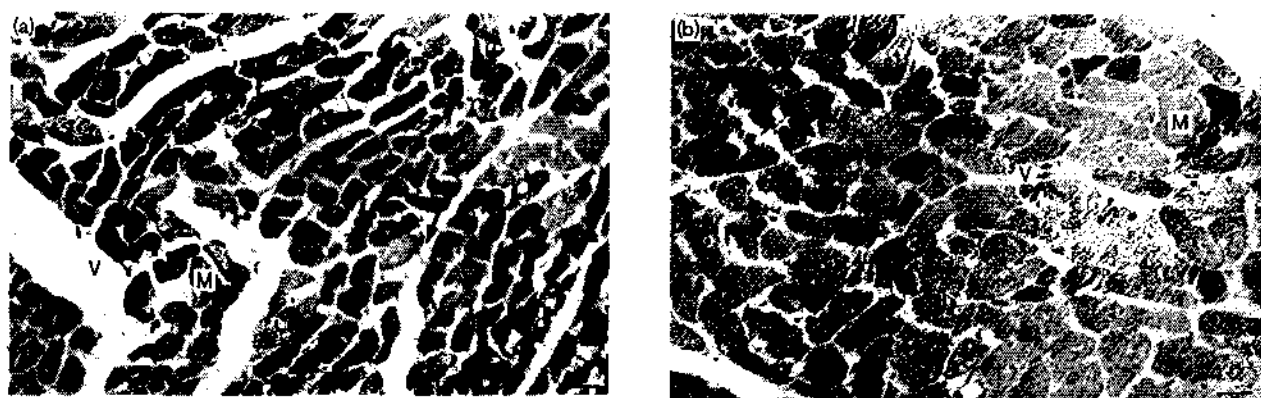
Mean arterial pressure was significantly increased in SHR rats compared with WKY rats (Table 1). Treatment with doxazosin (10 mg/kg body weight per day) for 15 days significantly reduced mean arterial pressure in SHR rats. Cardiac weight was greater in SHR rats than in WKY rats. Moreover, SHR rats demonstrated a slight although significant left ventricular hypertrophy when cardiac weight was normalized with respect to body weight. Light microscopy observation of the left ventricle showed larger cardiomyocytes in the left ventricle of SHR rats compared with those of WKY rats (Fig. 1).

Table 1 Mean arterial pressure (MAP) and parameters related to left ventricle (LV) hypertrophy in normotensive (WKY rats) and spontaneously hypertensive rats (SHRs) at 19 weeks of age. Effect of treatment with doxazosin

	WKY rats	SHRs	SHRs + DOX
MAP (mmHg)	121 ± 5	219 ± 8*	189 ± 7*
HW (g)	1.28 ± 0.08	1.67 ± 0.15*	1.64 ± 0.19*
CI (×10 ⁻³)	3.26 ± 0.04	4.80 ± 0.07*	4.95 ± 0.09*

Cardiac index (CI) is expressed as the ratio between heart weight (HW) and body weight (BW). Results are expressed as mean ± SEM of 16 rats from each experimental group. * *P* < 0.05 with respect to WKY rats. † *P* < 0.05 with respect to SHRs.

Fig. 1



Photomicrograph of 4 μ m representative cross-sections of the left ventricle of (a) Wistar-Kyoto (WKY) rats and (b) spontaneously hypertensive rats (SHRs) that have been stained with Masson's trichrome stain. The arrows point to collagen fibrils. Identification of collagen is even more obvious when the section is viewed in colour. (Original magnification $\times 350$). V, vessel; M, myocyte. Bar = 20 μ m.

Determination of the cardiomyocyte size, using a micrograduate lens, showed a size of: $18 \pm 2 \mu$ m for WKY rats and $28 \pm 1 \mu$ m for SHRs ($P < 0.05$). Doxazosin-treated SHRs showed values of cardiac weight and cardiac index close to those of untreated SHRs (Table 1). There were no significant differences in cardiomyocyte size between doxazosin-treated SHRs and untreated SHRs (27 ± 1 and 28 ± 1 mm, respectively).

Masson's trichromic extracellular matrix stain (Sigma), which stains collagen green, failed to demonstrate differences between SHRs and WKY rats (Fig. 1), suggesting that left ventricular hypertrophy in the 19-week-old SHRs, was related to cardiomyocyte hypertrophy rather than to an increase in extracellular matrix accumulation.

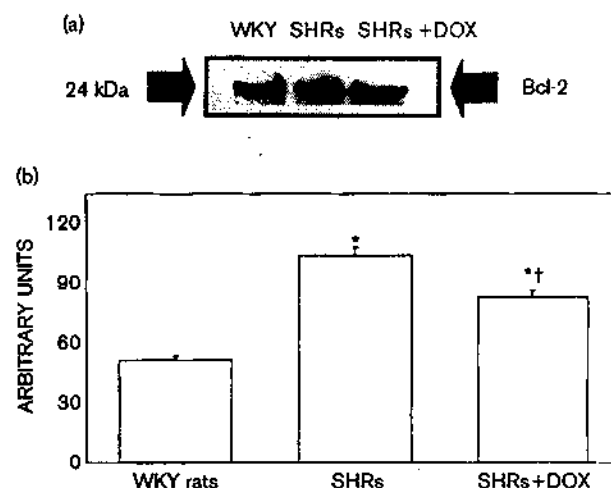
Expression of Bcl-2 and Bax proteins

Modifications of the levels of Bcl-2 and Bax protein expression were detected by Western blotting. SHRs showed a significantly higher expression of Bcl-2 protein than WKY rats (Fig. 2). Treatment with doxazosin tended to reduce Bcl-2 protein expression in the left ventricle of SHRs, and this was accompanied by a lower expression of Bax protein in SHRs than in WKY rats (Fig. 3). After treatment with doxazosin, Bax protein expression was significantly enhanced in the left ventricle of SHRs.

Verification that equal amounts of protein from each sample were loaded onto the gel was assessed by staining the gel Coomassie (Fig. 4).

The Bcl-2:Bax ratio, which has been considered as an apoptotic index [9,12,13], was significantly enhanced in

Fig. 2

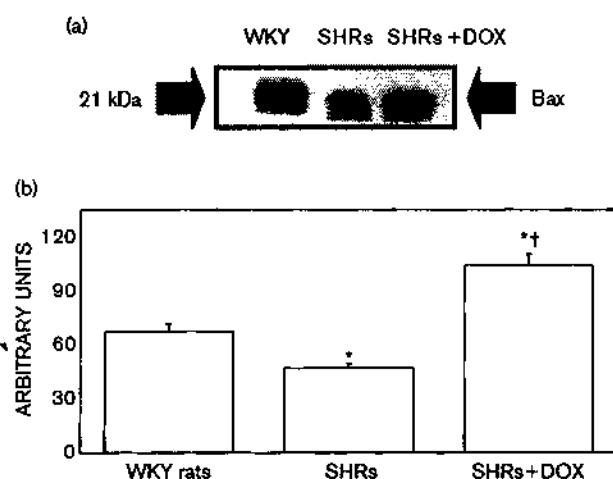


(a) Representative Western blot demonstrating the expression of Bcl-2 protein in the left ventricle of Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) treated or untreated with doxazosin (10 mg/kg per day) for 15 days. (b) Bar graph showing the densitometric scanning of the Western blot. Results are represented as means $\pm$ SEM. * $P < 0.05$ with respect to WKY rats. † $P < 0.05$ with respect to SHRs. DOX, doxazosin.

SHRs compared with WKY rats (2.2 ± 0.05 versus 0.89 ± 0.03 ; $P < 0.05$), indicating that Bcl-2 levels were higher than Bax levels in the left ventricle of SHRs. Treatment with doxazosin reduced the Bcl-2:Bax ratio in SHRs to a level similar to that found in WKY rats (0.83 ± 0.06).

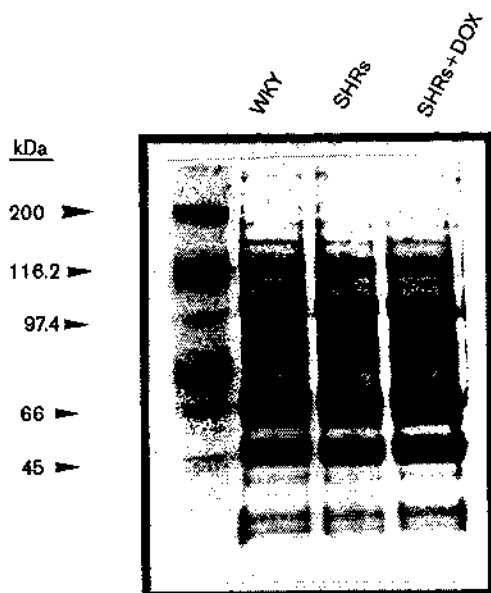
The results of the Western blot were further confirmed

Fig. 3



(a) Representative Western blot demonstrating the expression of Bax protein in the left ventricle of Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) treated or untreated with doxazosin (10 mg/kg per day) for 15 days. (b) Bar graph showing the densitometric scanning of the Western blot. Results are represented as means $\pm$ SEM. * $P < 0.05$ with respect to WKY rats. † $P < 0.05$ with respect to SHRs. DOX, doxazosin.

Fig. 4



Coomassie-stained gel to verify the amount of each sample as a control for loading. DOX, doxazosin; WKY, Wistar Kyoto rats; SHRs spontaneously hypertensive rats.

in the immunohistochemical studies. Expression of Bax protein was markedly lower in SHRs than in WKY rats (Fig. 5).

Doxazosin enhanced the expression of Bax protein in the left ventricle of SHRs. It is noteworthy that, whereas in the left ventricle of WKY rats Bax protein was present in most of the cardiomyocytes, in doxazosin-treated SHRs the pattern of expression of Bax protein seems to be focused to specific cardiomyocytes.

Immunolocalization of Bax protein demonstrated that the cells expressing the proteins were also positively stained with sarcomeric α -actin, indicating that they were cardiomyocytes (Fig. 5).

On the other hand, the immunohistochemical analysis also showed that the expression of Bcl-2 protein appeared to be increased in SHRs compared with the other experimental groups (data not shown).

Apoptotic cells and caspase-3-activity in the left ventricle of SHRs

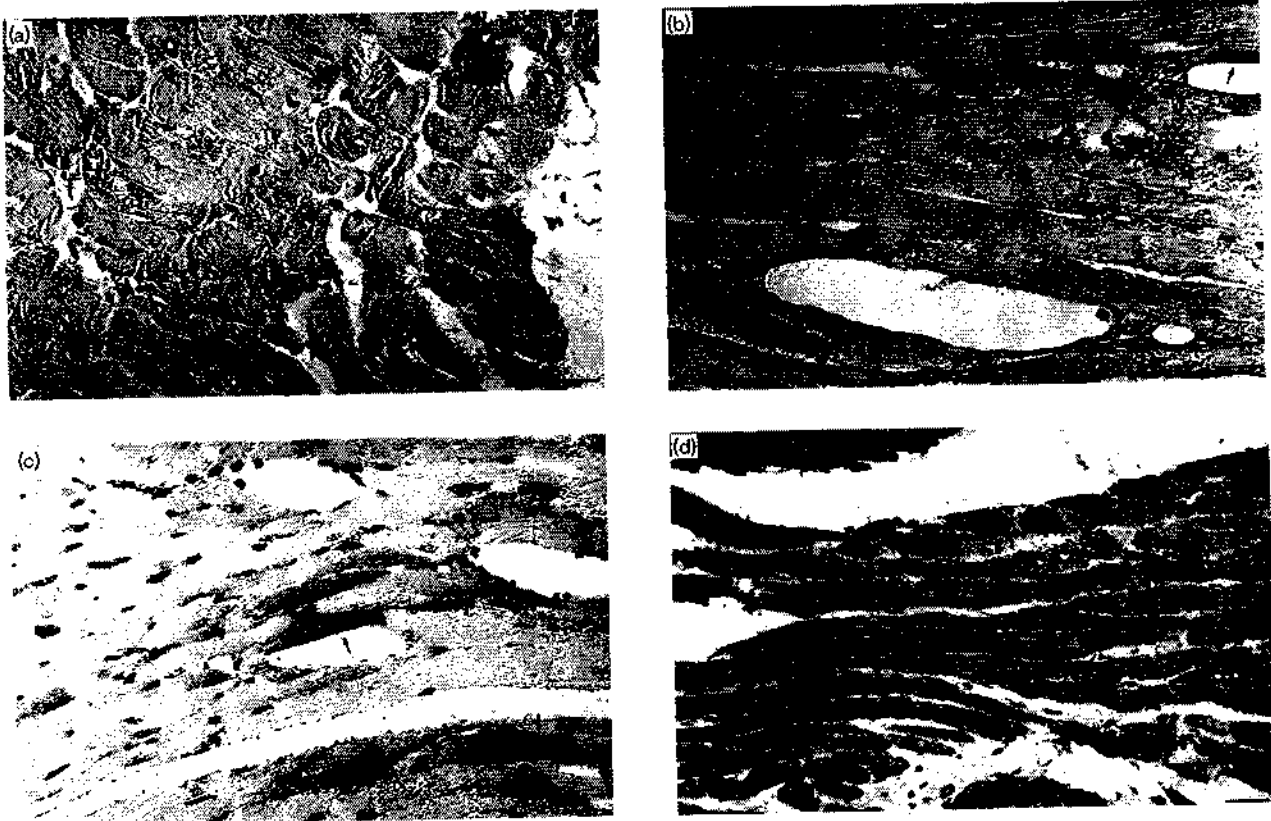
Cell apoptosis was measured by flow cytometry analysis of the DNA content in the left ventricles of the different groups of rats (Fig. 6). The percentage of cells with hypodiploid DNA content, indicative of cell apoptosis, was greater in the left ventricle of SHRs than in that of WKY rats (18.3 ± 0.03 versus $6.8 \pm 0.04\%$; $P < 0.05$). In the doxazosin-treated SHRs, the number of apoptotic cells tended to be reduced to the values measured in WKY rats (% apoptotic cells in doxazosin-treated SHRs: 10.4 ± 0.02 ; $P < 0.05$ with respect to SHRs).

In the left ventricle of WKY rats, the 85-kDa form of PARP was almost undetectable (Fig. 7). The SHRs also showed an increased level of the intact 116-kDa form of PARP compared with WKY rats, and this was accompanied by a marked increase in the levels of the 85-kDa PARP cleaved product (Fig. 7). In the left ventricle of doxazosin-treated SHRs the levels of the 85-kDa form were significantly reduced compared with those found in untreated SHRs.

Bax-Bcl-2 protein complexes in the left ventricle of SHRs

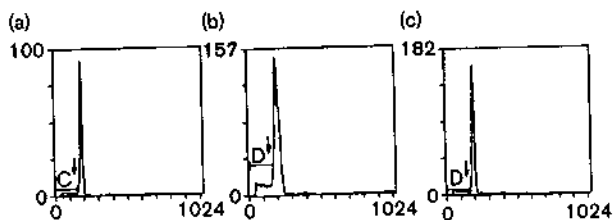
As paradoxical results were obtained with an increased number of apoptotic cells in the left ventricle of SHRs together with an anti-apoptotic state of the Bcl-2/Bax expression, we determined the amount of Bax protein bound to Bcl-2. The evidence taken from several studies suggests that Bax actively promotes cell death when it is bound to Bcl-2. The amount of Bax protein that formed complexes with Bcl-2 was higher in the left ventricle of SHRs than in either doxazosin-treated SHRs or WKY rats (Fig. 8, upper panel). Accordingly, the amount of Bax protein remaining in the supernatant

Fig. 5



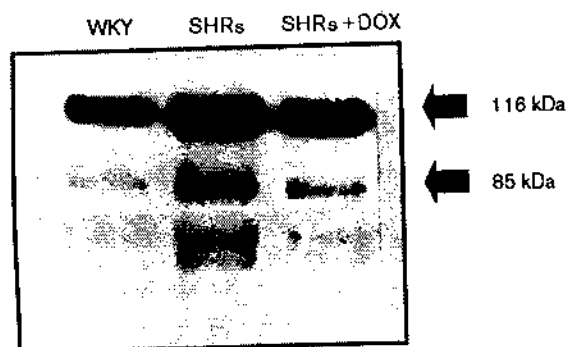
Four micrometre cross-sections of the left ventricles of (a) Wistar-Kyoto (WKY) rats, (b) spontaneously hypertensive rats (SHRs) and (c) doxazosin-treated SHRs. The expression of Bax protein was detected by a polyclonal antibody against Bax protein in paraffined section. Bax protein expression is indicated by arrows. (d) Positive immunostaining for sarcomeric α -actin indicating the presence of cardiomyocytes. (Original magnification $\times 400$). Bar = 20 μ m.

Fig. 6



Apoptotic cells in the left ventricle of (a) Wistar-Kyoto (WKY) rats, (b) spontaneously hypertensive (SHRs) and (c) doxazosin-treated SHRs (10 mg/kg body weight for 2 weeks). The number of apoptotic cells in the left ventricle was determined by flow cytometric analysis of the DNA content. The apoptotic cells appeared below the G_0/G_1 peak (arrow).

Fig. 7



Caspase-3 protease activity detected by the cleavage of the 85-kDa fragment of poly (ADP-ribose) polymerase (PARP). The 85-kDa fragment in the left ventricles of the different animals was detected by immunoblotting. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; DOX, doxazosin.

fraction after pelleting of the protein A-agarose immune complexes and, therefore, that failed to coprecipitate with Bcl-2 was lower in the left ventricle of SHR than in both doxazosin-treated SHRs and WKY rats (Fig. 8, lower panel).

Discussion

The present results suggest that in the left ventricle of SHRs there is an overexpression of the anti-apoptotic protein Bcl-2, which is accompanied by a reduction in the expression of the pro-apoptotic protein Bax. Consequently, the ratio Bcl-2:Bax was increased, suggesting that apoptosis was greatly inhibited in the left ventricle of SHRs. However, the number of apoptotic cells was higher in SHRs than in WKY rats. Moreover, we determined the PARP cleavage, a substrate of caspase-3, as index of cell apoptosis. Caspase-3 is a member of a family of aspartate-specific cysteine proteinases that plays a central role in the execution of the apoptotic programme [26]. The cleavage of PARP by caspase-3 has been implicated in the induction of apoptosis [27,28]. Whereas in the left ventricle of WKY rats the 85-kDa protein was almost undetectable, the left ventricle of SHRs showed a marked presence of the 85-kDa fragment of PARP, which suggested an increased caspase-3 protease activity. These changes were found in conditions in which blood pressure was increased but ventricular hypertrophy, although present, was not yet markedly pronounced.

Apparently, the present data could have paradoxical interpretations related to the coexistence in the left ventricle of SHRs of an increased Bcl-2:Bax ratio with both stimulation of caspase-3 protease activity and an

increased number of apoptotic cardiac cells. Other authors [9,12,13] have considered that the ratio Bcl-2:Bax is proportional to the susceptibility of the cells to apoptosis; however, recent growing evidence has suggested that Bax promotes cell death when it is bound to Bcl-2 [7,29]. This is supported by the fact that homodimers of Bcl-2 bind to the mitochondrial membrane and stabilize membrane permeability and the subsequent activation of caspases [6]. The stabilizing effects of Bcl-2 on mitochondrial permeability disappear when Bcl-2 is sequestered by Bax to form Bcl-2-Bax heterodimers [30]. Thus, a better parameter for the determination of the apoptotic state of a tissue could be the abundance of Bax protein that complexes with Bcl-2 protein.

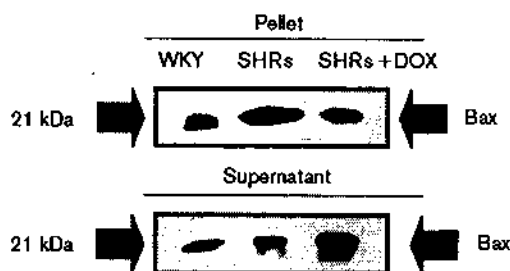
In the left ventricle of SHRs the formation of Bcl-2-Bax complexes was increased compared with WKY rats, which was in accordance with the flow cytometric results. The increase in the absolute Bcl-2:Bax ratio in the myocardium of SHRs could be interpreted as an attempt to maintain cell survival by both increasing the total amount of Bcl-2 protein and reducing Bax protein.

A reduction in the number of cardiomyocytes has been reported in the hypertrophied left ventricle of hypertensive patients [31]. With this in mind, Bing [32] has postulated that apoptosis may be a mechanism for the loss of viable cardiomyocytes, myocardial dysfunction and the transition from stable hypertrophy to heart failure associated with long-term chronic pressure overload. Moreover, it should be noted that increased apoptosis was present in failing SHR hearts compared with nonfailing SHR hearts [33]. Interestingly, in our study we detected changes in the Bax-Bcl-2 complexes and in the percentage of apoptotic cells before the rats showed a marked cardiac hypertrophy, suggesting that these changes take place in the early period of hypertrophy, long before the transition to heart failure.

Different clinical and experimental observations have suggested the role of α_1 -adrenergic stimulation in the development of left ventricle hypertrophy related to hypertension; Agabiti-Rosei *et al.* [34] have previously demonstrated that doxazosin, a selective α_1 -adrenoreceptor antagonist, reduced left ventricular mass in hypertensive patients. Therefore, we tested the effect of doxazosin on the expression of both Bcl-2 and Bax proteins in the left ventricle of SHRs.

Doxazosin modified Bcl-2 and Bax proteins in the left ventricle of SHRs towards a pro-apoptotic state. However, the formation of Bax-Bcl-2 complexes was decreased in the left ventricle of doxazosin-treated SHRs and this was accompanied by a reduction in the amounts of cardiac apoptotic cells and a reduction in the level of the 85-kDa fragment of PARP. Taken

Fig. 8



Representative Western blot showing Bax protein associated with Bcl-2 protein in the left ventricle of Wistar-Kyoto rats (WKY) rats, spontaneously hypertensive rats (SHRs) and doxazosin (DOX) treated SHRs (10 mg/kg body weight). The left ventricle lysates were immunoprecipitated with protein A-agarose-conjugated anti-Bcl-2 antibody and then immunoblotted with anti-Bax antibody. A typical autoradiograph of six different left ventricles from each experimental group is shown. The upper panel shows the Bax protein associated with Bcl-2. As a control, Bax protein was also determined in the supernatant fraction after immunoprecipitation with the anti-Bcl-2 antibody, which is indicative of the amount of Bax protein that failed to complex with Bcl-2 (lower panel).

together, these results suggested that doxazosin reduced the progression of cardiac apoptosis associated with hypertension.

A significant reduction of left ventricular mass has been demonstrated after long-term administration of doxazosin [34]. In our study, SHR were treated with doxazosin for only 15 days, which was insufficient to show changes in left ventricular hypertrophy, although it was enough to reduce blood pressure significantly. Fortuño *et al.* [35] have shown that chronic antihypertensive therapy, i.e. antagonizing angiotensin II type 1 (AT₁) receptors, normalizes the susceptibility of left ventricular cells to apoptotic stimuli and prevents the exaggerated apoptosis of left ventricular cells in SHR, and this was associated with the regression of ventricular hypertrophy in SHR. Similar findings have been described in SHR chronically treated with angiotensin converting enzyme (ACE) inhibitors [12]. Therefore, our results could suggest that changes in apoptosis of the left ventricle elicited by α_1 -blockade occur before modification in the left ventricle mass.

Our results also may indicate that the effects of doxazosin on Bcl-2 and Bax protein expression could be related to changes in haemodynamic forces. Studies have documented that apoptosis can be induced in cardiac cells by a variety of insults, including pressure overload [8]. The stretching of cardiac myocytes *in vitro*, which mimics an elevation of diastolic stress *in vivo*, induced apoptosis of these cells [35]. However, other evidence has demonstrated that changes in blood pressure were unrelated to apoptosis in the left ventricle of SHR [11,36]. Therefore, we could not rule out that Bcl-2 and Bax protein expression could be directly susceptible to α_1 -adrenoceptor antagonism.

In contrast to our results, Tea *et al.* [37] have proposed that other antihypertensive drugs such as calcium antagonists, ACE inhibitors and AT₁ receptors antagonists, which in the long term induced regression of cardiac hypertrophy, acutely increased apoptosis in SHR. Therefore, we cannot exclude different initial kinetic regulation of cardiomyocyte apoptosis by each specific factor involved in blood pressure regulation. However, with long-term antihypertensive treatment the inhibition of apoptosis seems to be associated with the regression of left ventricle hypertrophy [12,35].

In conclusion, our findings suggest that α_1 -adrenergic receptors may be involved in the observed increased apoptosis of left ventricular cells in SHR. Moreover, the evaluation of the Bcl-2:Bax expression ratio may not represent the apoptotic susceptibility of cardiac cells. A more reliable marker of the apoptotic state of the left ventricle cells was the evaluation of the abundance of Bax-Bcl-2 complexes.

Limitations of the study

The present study associated only the changes in the number of cardiac apoptotic cells and the cleavage of PARP, a substrate of caspase-3, with modifications in the formation of the Bax-Bcl-2 complexes. However, apoptosis is regulated by a number of molecular mechanisms that not only include Bax and Bcl-2 proteins, but also other factors such as tumor necrosis factor- α , Fas/Apo-1, c-myc and oxygen free radicals [38–40]. The role of these factors in apoptotic signalling pathways in cardiac cells is beginning to be investigated. Therefore, we can only speculate that modifications in the formation of Bax-Bcl-2 complexes in the left ventricle of SHR could determine the susceptibility of these cells to apoptosis. Additional studies are needed to clarify whether other promoters and inhibitors of apoptosis might be implicated in the above-described effects.

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