Doxazosin Induces Apoptosis in Cardiomyocytes Cultured In Vitro by a Mechanism That Is Independent of α_1-Adrenergic Blockade

José R. González-Juanatey, MD, PhD; María J. Iglesias, BS; Carlos Alcaide, BS; Roberto Piñeiro, BS; Francisca Lago, PhD

Background—The α_1-adrenoceptor–blocking antihypertensive doxazosin has been associated with increased risk of heart failure and is known to induce prostate cell apoptosis. We hypothesized that it might also induce apoptosis in cardiomyocytes.

Methods and Results—Hoechst dye vital staining and flow cytometry provided evidence that doxazosin induced apoptosis time- and dose-dependently in cardiomyocytes of the HL-1 cell line. TUNEL assays and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability test confirmed that doxazosin induced DNA damage and cell death in these cells. MTT tests showed that doxazosin treatment decreased cell viability in primary cultures of neonatal rat cardiomyocytes, and Hoechst dye vital staining demonstrated doxazosin-induced apoptosis in primary cultures of human adult cardiomyocytes. The proapoptotic effect of doxazosin on cardiomyocytes seems not to depend on α_1 blockade, because it was not modified by cotreatment with α- or β-adrenergic agonists or with the irreversible α_1-blocker phenoxybenzamine and because doxazosin also decreased the viability of NIH 3T3 cells, which lack α_1-adrenoceptors. It also does not involve calcineurin, being unaffected by the presence of the calcineurin inhibitors cyclosporin A and FK506. Three other α_1-blockers were also investigated; prazosin was proapoptotic, like doxazosin, but 5-methylurapidil and terazosin were not.

Conclusions—The α_1-blockers doxazosin and prazosin induce the apoptosis of cardiomyocytes cultured in vitro by a mechanism that is independent of α_1 blockade and calcineurin. **(*Circulation*. 2003;107:●●●-●●●.)**

Key Words: apoptosis ■ hypertension ■ myocytes

The antihypertensive doxazosin, an α_1-adrenoceptor blocker, was found in the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) study to be associated with greater risk of cardiovascular accidents than chlorthalidone.[1] The biological basis of this association has not been elucidated, but it is known that doxazosin induces the apoptosis of prostate cancer cells[2–5] and halts the cell cycle of human coronary smooth muscle cells.[6,7] In the present study, we used a variety of tests to investigate whether doxazosin also induces the apoptosis of various types of cultured cardiomyocytes. We found that it does and that this action is independent of α_1 blockade and calcineurin. Of the 3 other α_1-blockers investigated, prazosin was also proapoptotic, but 5-methylurapidil and terazosin were not.

Methods

All products were from Sigma Chemical Co unless otherwise stated.

Cells

HL-1 cardiomyocytes (a gift from Dr W.C. Claycomb, Louisiana State University Medical Center) were cultured on fibronectin-covered plates with ExCell 320 (JRH Biosciences Ltd) supplemented with FBS (from Life Technologies Ltd), insulin, norepinephrine, endothelial cell growth supplement (Upstate Biotechnology Inc), and retinoic acid.[8] Mouse fibroblast cells (NIH 3T3) were grown in DMEM supplemented with 10% FCS (Life Technologies, Ltd) and L-glutamine. Primary cultures of rat neonatal cardiomyocytes were established as follows: The hearts of 1-day-old Sprague-Dawley rats were digested with collagenase and pancreatin at 37°C in 3 30-minute digestion cycles, cells were centrifuged from the pooled supernatants, and fibroblasts were removed by differential seeding with incubation for 4 hours at 37°C in newborn calf serum (Life Technologies, Ltd); the cardiomyocytes were seeded at a density of 50 000/cm^2 in gelatin-coated plates containing DMEM/M199 medium (Life Technologies, Ltd) supplemented with horse serum (Life Technologies, Ltd), FCS, L-glutamine, and antibiotics. Primary cultures of human cardiomyocytes were established as follows: In surgery requiring cardiopulmonary bypass, a catheter is placed in the right atrium. The catheter passes via an opening made by excising part of the right atrial appendage. The excised tissue is normally discarded. These human right atrial appendages were minced in small pieces and digested at 37°C in 3 5-minute cycles with PBS containing 0.25% trypsin, 0.15% collagenase, and 0.02% glucose, and cells were centrifuged from the pooled supernatants for 5 minutes at 580*g* and cultured in Iscove's modified Dulbecco's

Received July 25, 2002; revision received September 10, 2002; accepted September 13, 2002.

From the Cardiology Research Unit, University Clinical Hospital, Santiago de Compostela, Spain.

Correspondence to Dra. Francisca Lago Paz, Laboratorio de Investigación 1, Planta Baja, Area de Investigación y Docencia, Hospital Clínico Universitario de Santiago de Compostela, Travesía Choupana s/n, 15706 Santiago de Compostela, Spain. E-mail frlago@usc.es

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/01.CIR.0000043803.20822.D1**

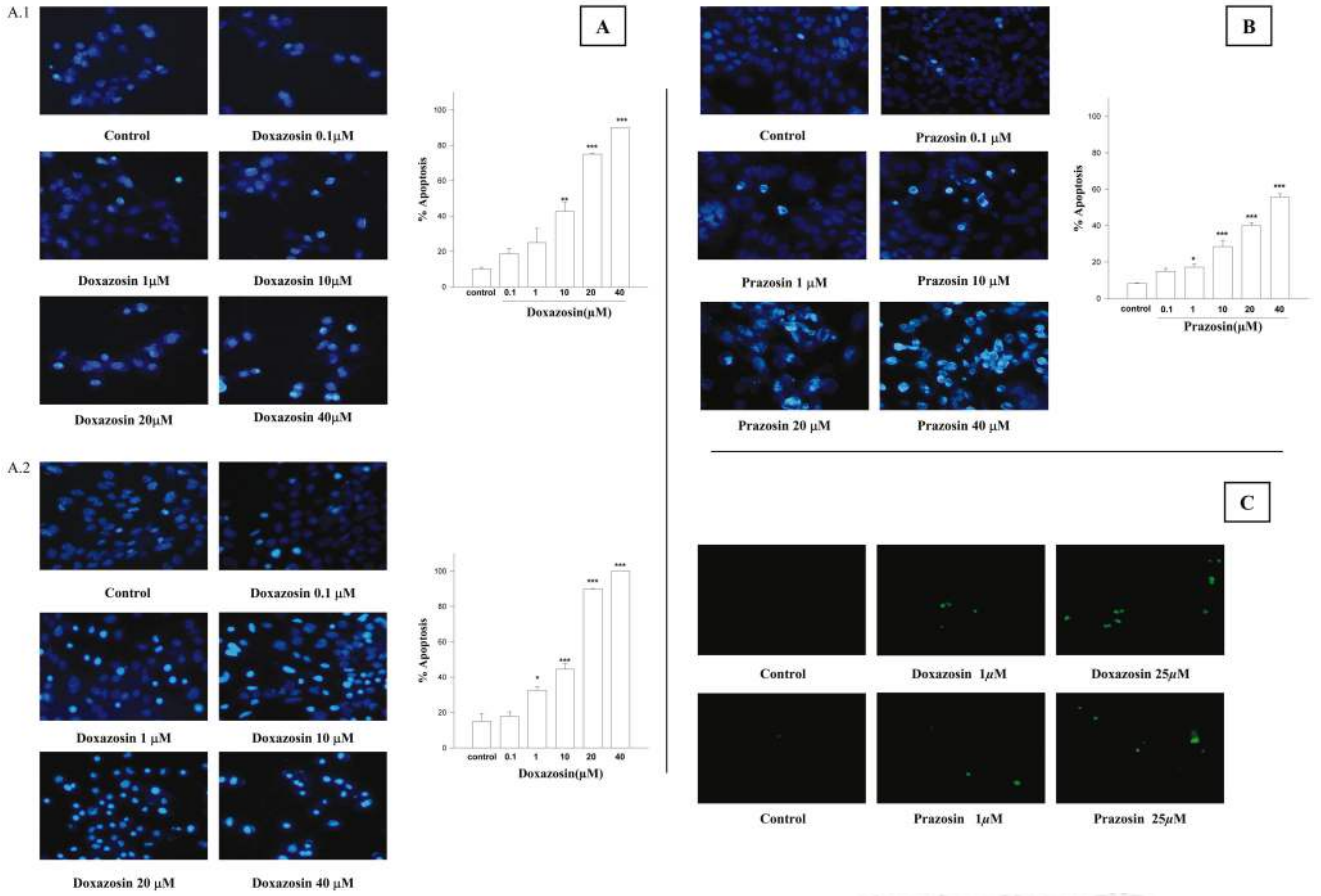

Figure 1. Doxazosin and prazosin induce apoptosis in HL-1 cardiomyocytes. A, Apoptosis (shown by brilliant blue nuclear fluorescence and fragmentation) revealed by staining with Hoechst 33258 dye after treatment with 0.1 to 40 μmol/L doxazosin for 12 hours (A.1) or 48 hours (A.2); quantitative results of 3 independent experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$ relative to controls) and fluorescence microphotographs (×400) of one typical experiment. B, As for panel A.2, but with prazosin instead of doxazosin. C, Fluorescence microphotographs (×200) corresponding to TUNEL assays of cells treated for 48 hours with doxazosin or prazosin (1 to 25 μmol/L); green fluorescence shows DNA damage.

medium (Life Technologies, Ltd) supplemented with 10% FBS, L-glutamine, and antibiotics.

Hoechst Vital Staining

Cardiomyocytes (10^4) were treated for 12 or 48 hours with 0.1 to 40 μmol/L doxazosin (a gift from Pfizer Pharmaceuticals, New York, NY) or for 48 hours with 0.1 to 40 μmol/L prazosin and were then incubated for 45 minutes at 37°C in Hoechst 33258 dye.

TUNEL Assays

TUNEL assays were performed using the In Situ Cell Death Detection Kit (Fluorescein) from Boehringer Mannheim Corp.

MTT Viability Assay

Cardiomyocytes (10^4 per treatment) were treated for 48 hours with 0.1 to 50 μmol/L doxazosin, prazosin, terazosin, or 5-methylurapidil and 4 hours before the expiry of this period with 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). After overnight incubation at 37°C, absorbance at 550 to 600 nm was measured.

Cell-Cycle Analysis

HL-1 cells (10^6 per treatment) were treated for 72 hours with 0.1 to 10 μmol/L doxazosin with or without FBS (15%); 4% paraformaldehyde was added; and after 10 minutes, the cells were incubated overnight in 70% ethanol at −20°C. The cell pellets were then incubated for 1 hour in a solution containing 1 mg/mL RNase and 5 μg/mL propidium iodide, after which flow cytometry was performed in a FACSCALIBUR apparatus (Becton & Dickinson) using the CellQuest program. Apoptosis was measured as the percentage of DNA in the hypodiploid (sub-G_0/G_1) peak.

Statistical Analysis

Results are mean±SEM of those of the stated number of independent experiments (n). The statistical significance of differences between means was estimated using ANOVA followed by Student Newman-Keuls multiple comparison test.

Results

Doxazosin- and Prazosin-Induced Apoptosis of HL-1 Cells

Relative to controls, doxazosin and prazosin increased apoptosis among HL-1 cells, and the increase was dose and time dependent, as assessed by Hoechst dye vital staining (Figures 1A and 1B). DNA damage in cardiomyocytes treated with doxazosin and with prazosin was confirmed by TUNEL assays (Figure 1C).

Figure 2A shows the percentages of apoptotic, G_0/G_1-phase, and S/G_2/M-phase cells measured by flow cytometry after 72 hours in 1 of 3 independent experiments, the other 2

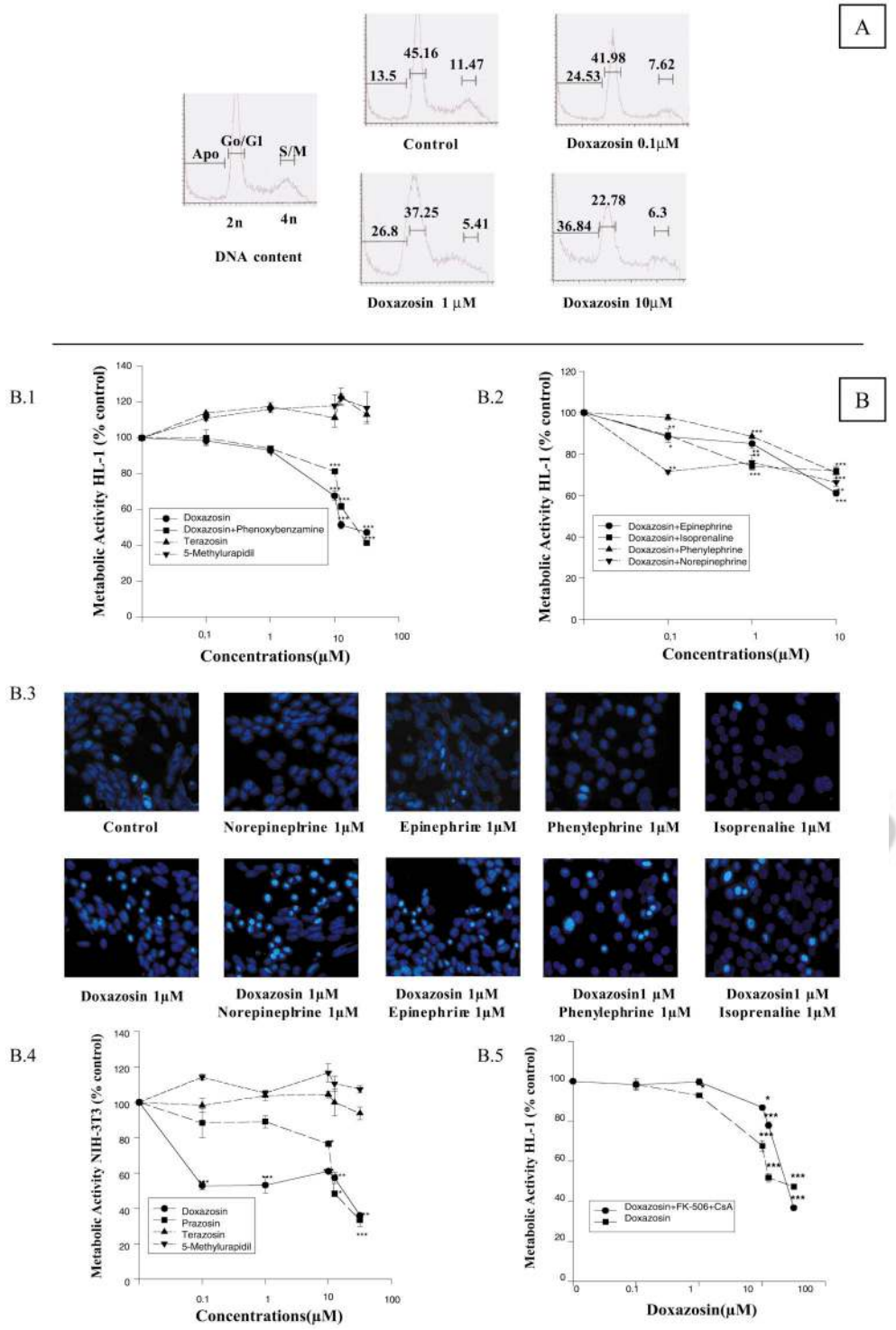

Figure 2. Effect of doxazosin on the viability of HL-1 cardiomyocytes or NIH 3T3 cells. A, FACS analysis of HL-1 cells treated with doxazosin for 72 hours and stained with propidium iodide (results of 1 of 3 independent experiments), showing numbers of cells plotted against DNA content; values are percentages of cells that are apoptotic (Apo) or in the G_0/G_1 (2n) or S/M (4n) phases. B, Doxazosin-induced cell death of HL-1 cells is independent of α_1-adrenoceptor blockade and calcineurin activity. B.1, Prior exposure to 1 μmol/L phenoxybenzamine for 4 hours did not alter the MTT-evaluated effects on HL-1 cells of 48 hours of exposure to 0.1 to 50 μmol/L doxazosin; terazosin and 5-methylurapidil did not decrease HL-1 cell viability. B.2, MTT-assessed cell death of HL-1 cells after 48 hours of incubation with doxazosin (0.1 to 10 μmol/L) was not reduced by the presence of equal concentrations of the α- and β-agonists norepinephrine and epinephrine, the α-agonist phenylephrine, or the β-agonist isoprenaline. B.3, Fluorescence microphotographs ($\times$400) of HL-1 cells treated for 48 hours with doxazosin and/or epinephrine, norepinephrine, isoprenaline, or phenylephrine (1 μmol/L in each case) before Hoechst staining. B.4, Treatment for 24 hours with 0.1 to 50 μmol/L doxazosin or prazosin induced the dose-dependent cell death of NIH 3T3 cells, as assessed by the MTT test; terazosin and 5-methylurapidil did not affect NIH 3T3 viability. B.5, Presence of the calcineurin inhibitors cyclosporin A (CsA, 3 μmol/L) and FK506 (150 ng/mL) did not prevent the dose-dependent induction of HL-1 cell death by 48 hours of exposure to 0.1 to 50 μmol/L doxazosin, as assessed by the MTT test. Statistical significance of differences in B.1, B.2, B.4, and B.5 (n=3): $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

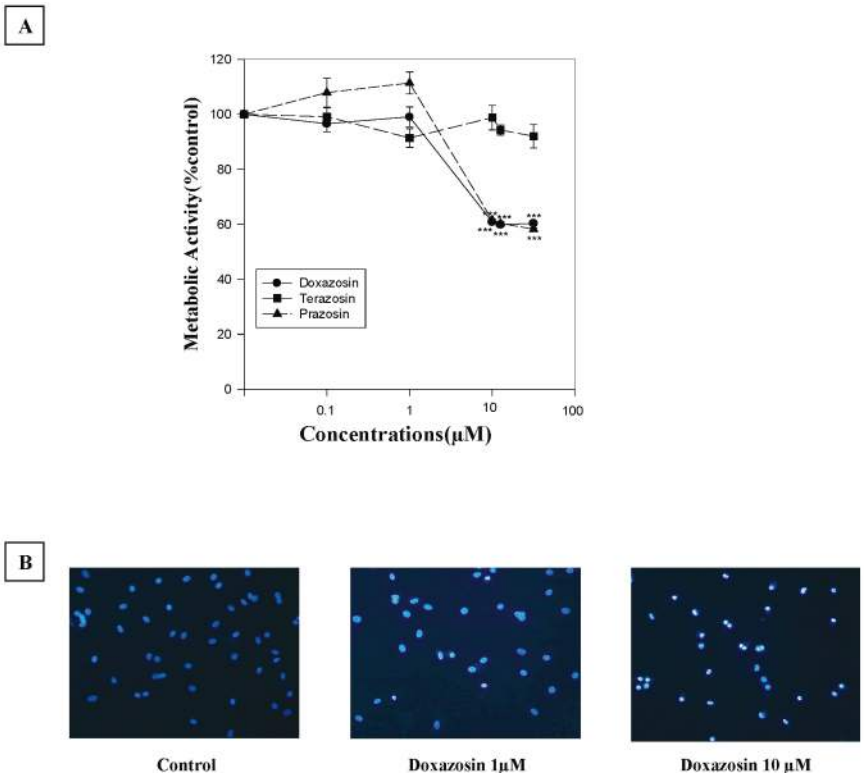

Figure 3. Effect of doxazosin on primary cultures of cardiomyocytes. A, Effect of doxazosin, prazosin, and terazosin on primary cultures of neonatal rat cardiomyocytes. Treatment for 48 hours with doxazosin or prazosin decreased MTT-assessed cell viability, whereas terazosin had no effect. Statistical significance of differences (n=3): *$P<0.05$; **$P<0.01$; ***$P<0.001$. B, Effect of doxazosin on human cardiomyocytes cultured in vitro. Fluorescence microphotographs (×400) show apoptosis (brilliant blue nuclear fluorescence) after treatment with 1 to 10 μmol/L doxazosin for 48 hours and subsequent staining with Hoechst 33258 dye.

of which afforded similar results. Apoptosis was 13.5%, 24.53%, and 36.84% among control cells and cells treated with 0.1 and 10 μmol/L doxazosin, respectively, in keeping with which, the percentages of $S/G_2/M$-phase and G_0/G_1-phase cells fell with increasing doxazosin concentration from 11.47% and 45.16%, respectively, among controls to 6.3% and 22.78%, respectively, among cells treated with 10 μmol/L doxazosin.

Independence of α_1-Adrenoceptor Blockade and Calcineurin

In MTT viability tests, doxazosin induced loss of cells and metabolic activity, and this effect was not altered by prior exposure to the irreversible α-blocker phenoxybenzamine, which did not itself reduce cell viability (Figure 2B1); prazosin behaved similarly (results not shown). Terazosin and 5-methylurapidil did not decrease cell viability (Figure 2B1). Cell death was not affected by the presence of α- and β-adrenergic agonists (epinephrine, norepinephrine, phenylephrine, and isoprenaline, equimolar with doxazosin) (Figures 2B2 and 2B3). Doxazosin and prazosin also reduced the MTT-assessed viability of NIH 3T3 cells, which have no α-adrenoceptors, whereas terazosin and 5-methylurapidil had no effect (Figure 2B4).

In view of reports that in cardiomyocytes the Ca^{2+}/calmodulin-activated phosphatase calcineurin can act both proapoptotically[9] and antiapoptotically,[10] we investigated whether it is involved in the action of doxazosin on cardiomyocytes. The fact that it is not is shown by the action of doxazosin being unaffected by the presence of the calcineurin inhibitors cyclosporin A and FK506 (Figure 2B5).

Reduction of Viability of Human and Neonatal Rat Cardiomyocytes in Primary Cultures

In MTT viability tests, doxazosin and prazosin induced loss of cells and metabolic activity in primary cultures of neonatal rat cardiomyocytes, whereas terazosin had no effect (Figure 3A). Hoechst dye vital staining showed that doxazosin was also proapoptotic for primary cultures of human cardiomyocytes (Figure 3B).

Discussion

In ALLHAT,[1] the main reason for early withdrawal of doxazosin from the study by the National Heart, Lung, and Blood Institute (NHLBI) was the finding that it was associated with a greater risk of cardiovascular accidents, heart failure in particular, than the diuretic chlorthalidone. Previously, the Vasodilator Heart Failure Trial 1 (V-HeFT) had found that the α_1-blocker prazosin was associated with greater mortality among heart failure patients than were other vasodilators.[11] Given the hemodynamic, metabolic, and vasodilatory benefits afforded by α_1-blockers, these findings were surprising, although by the time of the ALLHAT report it was becoming known that doxazosin halts the cell cycle of human coronary smooth muscle cells[6,7] and that it induces the apoptosis of prostate cancer cells.[2–5] In the present study, we investigated whether the α_1-blockers doxazosin, prazosin, 5-methylurapidil, and terazosin induce the apoptosis of HL-1 cells, which derive from murine atrial cardiomyocytes and were considered to constitute a suitable in vitro model because they maintain a heart-specific phenotype[8] and normally feature all of the cell-surface and intracellular components required for an α_1-adrenergic response.[12] We also used

primary cultures of human and neonatal rat cardiomyocytes to confirm the results obtained with HL-1 cells.

We found that both doxazosin and prazosin induced the apoptosis of cultured cardiomyocytes dose-dependently over the range 0.1 to 50 μmol/L, whereas terazosin and 5-methylurapidil had no effect on cultured cardiomyocytes' viability. The effect of doxazosin was already considerable at the 0.1-μmol/L level when assessed by flow cytometry after 72 hours and was statistically significant at the 1-μmol/L level when assessed after 48 hours by Hoechst dye vital staining. A concentration of 1 μmol/L in vitro is considered[4,7] to ensure intracellular concentrations similar to those achieved in vivo by therapeutic doses (in patients, serum doxazosin concentration reaches 0.122 μmol/L with an 8-mg dose and 0.244 μmol/L with a 16-mg dose).[13] It should be noted, moreover, that in our experiments, doxazosin and prazosin, both of which are usually used for long-term treatment of hypertensive patients, caused apoptosis in vitro within just a few days.

As in the case of other cells,[4,5,7] the proapoptotic action of doxazosin and prazosin on cardiomyocytes did not depend on α_1-blocking activity, because it was not reduced by the presence of α- or β-adrenergic agonists or prevented by pretreatment with the irreversible α-blocker phenoxybenzamine (which did not itself reduce cell viability), and doxazosin and prazosin also induced the death of NIH 3T3 cells, which lack α_1-adrenoceptors. Nor did its mechanism seem to involve the Ca^{2+}/calmodulin-activated phosphatase calcineurin (which is known to be involved in cardiomyocyte apoptosis regulation),[9,10,14] because it was unaffected by the presence of the calcineurin inhibitors cyclosporin A and FK506. It is possible that the induction of apoptosis by doxazosin and prazosin may be related to the finding that prazosin inhibits the synthesis of heat-shock proteins,[15] but in exploratory experiments we have found that HL-1 cardiomyocytes show no change in HSP70 expression after doxazosin treatment (results not shown). Another possibility that we are presently investigating is that the induction of apoptosis by doxazosin and prazosin may involve alteration of integrin-mediated binding to extracellular fibronectin.

The results of the present study are in keeping with reports of apoptosis induction by doxazosin in other cells[2–7] and may well explain the cardiovascular effects observed in the ALLHAT and V-HeFT studies, particularly the increased incidence of heart failure, because apoptosis is known to occur in the early stages of myocardial dysfunction and to contribute to progressive cardiomyocyte loss in heart failure.[16] It may be hypothesized that the induction of cardiomyocyte apoptosis by doxazosin or prazosin accelerates the early stages of these clinical processes. Indeed, clinical effects may be felt before full apoptosis. Because apoptosis involves the activation of protein-cleaving caspases, cleavage of cardiomyocyte myofilaments by caspases may lead to contractile dysfunction before cell death.[17] The clinical implications of our results nevertheless remain to be elucidated, although we note, finally, that our findings may be relevant not only to treatment of hypertension but also to the consideration of doxazosin for treatment of benign prostatic hyperplasia.

Acknowledgments

This research was supported by Pfizer Inc. through its International Cardura Competitive Awards Program.

References

1. ALLHAT Research Group. Major cardiovascular events in hypertensive patients randomized to doxazosin vs chlorthalidone: the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). *JAMA*. 2000;283:1967–1975.
2. Chon JK, Borkowski A, Partin AW, et al. α_1 adrenoceptor antagonists terazosin and doxazosin induce prostate apoptosis without affecting cell proliferation in patients with benign prostatic hyperplasia. *J Urol*. 1999; 161:2002–2008.
3. Kyprianou N, Chon J, Benning CM. Effects of α_1-adrenoceptor antagonists on cell proliferation and apoptosis in the prostate: therapeutic implications in prostatic disease. *Prostate Suppl*. 2000;9:34–41.
4. Kyprianou N, Benning C. Suppression of human prostate cancer cell growth by α_1-adrenoceptor antagonists doxazosin and terazosin via induction of apoptosis. *Cancer Res*. 2000;60:4550–4555.
5. Benning CN, Kyprianou N. Quinazoline-derived α_1-adrenoceptor antagonists induce prostate cancer cell apoptosis via an α_1-adrenoceptor-independent action. *Cancer Res*. 2002;62:597–602.
6. Hu ZW, Shi XY, Hoffman BB. Doxazosin inhibits proliferation and migration of human vascular smooth-muscle cells independent of α_1 adrenergic receptor antagonism. *J Cardiovasc Pharmacol*. 1998;31:833–839.
7. Kintscher U, Wakino S, Kim S, et al. Doxazosin inhibits retinoblastoma protein phosphorylation and G1-S transition in human coronary smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2000;20:1216–1224.
8. Claycomb WC, Lanson NA, Stallworth BS, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A*. 1998;95:2979–2984.
9. Saito S, Hiroi Y, Zou Y, et al. β-adrenergic pathway induces apoptosis through calcineurin activation in cardiac myocytes. *J Biol Chem*. 2000; 275:34528–34533.
10. De Windt LJ, Lim HW, Taigen T, et al. Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis in vivo and in vitro: an apoptosis-independent model of dilated heart failure. *Circ Res*. 2000;86: 255–263.
11. Cohn JN, Archibald DG, Ziescha S, et al. Effect of vasodilator therapy on mortality in chronic congestive heart failure: results of a Veterans Administration cooperative study. *N Engl J Med*. 1986;314:1547–1552.
12. McWhinney CD, Hansen C, Robishaw JD. α_1-Adrenergic signaling in a cardiac murine atrial myocyte (HL-1) cell line. *Mol Cell Biochem*. 2000; 214:111–119.
13. Frick MH, Halttunen P, Himanen P, et al. A long-term double-blind comparison of doxazosin and atenolol in patients with mild to moderate essential hypertension. *Br J Clin Pharmacol*. 1986;21:55S–62S.
14. Molkentin JD. Calcineurin, mitochondrial membrane potential, and cardiomyocyte apoptosis. *Circ Res*. 2001;88:1220–1222.
15. Matz JM, La Voi KP, Moen RJ, et al. Cold-induced heat shock protein expression in rat aorta and brown adipose tissue. *Physiol Behav*. 1996; 60:1369–1374.
16. Kang PM, Izumo S. Apoptosis and heart failure: a critical review of the literature. *Circ Res*. 2000;86:1107–1113.
17. Communal C, Sumandea M, de Tombe P, et al. Functional consequences of caspase activation in cardiac myocytes. *Proc Natl Acad Sci U S A*. 2002;99:6252–6256.