JPP Journal of Pharmacy and Pharmacology

JPP 2009, 61: 503–510

Received October 15, 2008
Accepted January 7, 2009
DOI 10.1211/jpp/61.04.0013
ISSN 0022-3573

Research Paper

Synergism of simvastatin with losartan prevents angiotensin II-induced cardiomyocyte apoptosis *in vitro*

Jian Xu[a,b], Xiong-wen Lü[a], Yan Huang[a], Peng-li Zhu[a] and Jun Li[a]

[a]School of Pharmacy, Anhui Medical University, Hefei and [b]Department of Cardiology, Anhui Provincial Hospital, Hefei, China

Abstract

Objectives Increasing evidence suggests that cardiomyocyte apoptosis has an important role in the transition from compensatory cardiac remodelling to heart failure. The synergistic effect of statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) and angiotensin II (Ang II) type 1 receptor antagonists reduces the incidence of cardiovascular events. However, the anti-apoptotic potential of the synergism between losartan and simvastatin in heart failure remains unexplored. Here, we demonstrate that Ang II-induced apoptosis is prevented by losartan and simvastatin in neonatal cardiomyocytes.

Methods The in-vitro cardiomyocyte apoptosis model was established by co-culturing neonate rat cardiomyocytes with Ang II. Cell viability was analysed by the MTT assay. Cell apoptosis was evaluated using fluorescence microscopy and flow cytometry. Apoptosis-related proteins Bax and Bcl-2 expressions were measured by flow cytometry detection.

Key findings Incubation with 10^{-7} M Ang II for 48 h increased cardiomyocyte apoptosis and decreased cell viability. Losartan (10^{-5} M) and simvastatin (10^{-5} M), either alone or in combination, significantly decreased Ang II-induced cardiomyocyte apoptosis and increased cell viability. The q values calculated by the probability sum test were 1.31 for cardiomyocyte apoptosis and 1.21 for cell viability. Ang II induced a significant increase in Bax protein expression, whereas Bcl-2 protein expression was decreased. Losartan alone or in combination with simvastatin blocked the increased Bax expression and increased Bcl-2 expression. However, simvastatin had no such effect.

Conclusions Our data provide the first evidence that synergism of simvastatin with losartan prevents angiotensin II-induced cardiomyocyte apoptosis *in vitro*. Synergism between simvastatin and losartan may provide a new therapeutic approach to the prevention of cardiac remodelling.

Keywords angiotensin II; Bax; Bcl-2; cardiomyocyte apoptosis; losartan; simvastatin

Introduction

Apoptosis is an active mechanism by which cells respond to insults by triggering a programme of gene-regulated cell death. It has been increasingly recognised that apoptosis plays an important role in normal development and in pathology in a wide variety of tissues.[1,2] Cardiomyocyte apoptosis may be a critical factor during the transition from compensatory hypertrophy to heart failure.[3,4] Inhibition of cardiomyocyte apoptosis may prevent and restrain left ventricular hypertrophy and heart failure.[5] The mechanisms of cardiomyocyte apoptosis include extrinsic factors and various cellular signalling pathways. Bcl-2 expression has been proposed as an important marker of myocardial cell survival probability.[6] Studies have shown that the balance of Bcl-2 to Bax plays a central role in ischaemia-dependent apoptosis, while angiotensin (Ang) II may be one of the major causes of receptor-mediated apoptosis.[7]

Ang II, a key molecule for cardiovascular regulation, acts as a candidate entraining signal, altering circadian expressions of central clock genes through the Ang II type 1 (AT_1) receptors. It is well recognised that cardiac hypertrophy is mediated by AT_1 receptors.[8,9] Studies have also shown that AT_1 receptors play an important pro-apoptotic role in cardiomyocytes of both neonatal and adult rats.[10–13] Therefore, the development of new agents that inhibit Ang II-induced cardiomyocyte apoptosis may be beneficial for the treatment of cardiac dysfunction.

Correspondence: Professor Jun Li, School of Pharmacy, Anhui Medical University, Hefei 230001, China.
E-mail: lijun@ahmu.edu.cn

Losartan, a selective AT_1 receptor antagonist, is widely used clinically to control blood pressure. Several studies have shown that losartan has beneficial effects in attenuating cardiac dysfunction, including the suppression of the later development of hypertrophy and heart failure, through inhibiting protein kinase C activation in the remote non-ischaemic area of infarcted rat heart, delaying the development of heart failure in dogs with asymptomatic left ventricular dysfunction, prolonging survival in dilated cardiomyopathy, and retardation of pressure-overload hypertrophy.[14–17] Statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) are widely used clinically as lipid-lowering agents. Statins have been reported to prevent the development of heart failure in ischaemic heart disease and after myocardial infarction.[18–20] Losartan and simvastatin are widely used, often in combination, in the treatment of cardiovascular disease. Recently, the importance of combination therapy has been recognised in the treatment of adriamycin-induced nephropathy, coronary heart disease, myocardial infarction and heart failure.[21–25] However, the synergism between losartan and simvastatin in cardiomyocyte apoptosis has not been investigated.

Experiments using cultured neonatal and adult cardiomyocytes have demonstrated that apoptosis can be stimulated *in vitro* by several endogenous peptides such as Ang II (a natural effector of myocardial apoptosis).[26] However, the molecular mechanisms that result in cardiomyocyte apoptosis after exposure to Ang II remain largely unknown. In this study, we first investigated the synergism between losartan and simvastatin on apoptosis induced by Ang II. To further explore the possible pharmacological mechanisms of this synergism, we analysed Bax and Bcl-2 protein expression in cultured neonatal cardiomyocytes.

Materials and Methods

Materials

Ang II, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide, fluorescein isothiocyanate (FITC)–Annexin V and Dulbecco's modified essential medium were purchased from Sigma (St Louis, MO, US). Fetal bovine serum (FBS), cardiac myocyte growth supplement (CMGS) and penicillin/streptomycin solution were bought from ScienCell Research Laboratories (Carlsbad, CA, US). Anti-Bax and anti-Bcl-2 antibodies were bought from Santa Cruz Biotechnology (Santa Cruz, CA, US). Losartan and simvastatin were purchased from Merck, Sharp & Dohme Co., Hangzhou, China).

Losartan was dissolved in DMSO. As cardiomyocytes lack the lactonases required to convert simvastatin to its open acid active form, this was done chemically. Briefly, 20 mg simvastatin was suspended in 0.4 ml ethanol and 2.4 ml 0.1 M NaOH added. The solution was heated to 50°C for 2 h. To this heated solution we added 3.6 ml of an aqueous solution containing 81 mM Na_2HPO_4 and 15 mM NaH_2PO_4. The solution was heated to 40°C for 30 min, and the pH adjusted to 7.3 using concentrated HCl. The control was DMSO diluted to the same concentration as the simvastatin solution.

Cell culture and treatment

Rat cardiac myocytes isolated from neonate rat heart (ventricle) and cryopreserved in primary cultures were delivered frozen (ScienCell Research Laboratories). Each vial contained at least 1×10^6 cells in 1 ml volume. After resuscitation, cells were cultured; they were used when they had reached at least 90% confluency.

Angiotensin-II-induced apoptosis and drug administration

Cell purity was confirmed by immunocytochemistry using antibodies against alpha-actin (data not shown). The cultures were free of other contaminating cell types (>99% purity). Cardiomyocytes were plated onto laminin-precoated (0.5 mg/cm^2) culture plates at a density of 1.5×10^4 cells/cm^2 and incubated for 4 h at 37°C in a 5% CO_2 humidified atmosphere. The cardiomyocyte culture medium consisted of 500 ml basal medium, 25 ml FBS, 5 ml CMGS and 5 ml penicillin/streptomycin solution. Non-attached cells were discarded after this period and fresh culture medium was added. Cardiomyocytes were collected and replated (2×10^5/ml) into 96-well plates for MTT assay, and into 24-well plates for morphological assessment and flow cytometry. Briefly, after incubation for 24 h, Ang II was added, diluted in the culture medium at 10^{-7} M, for another 48 h. After Ang II treatment, cells were analysed for apoptosis. In some wells, losartan and simvastatin were added, diluted to 10^{-5} M in the medium, and Ang II was added after 1 h of incubation.

Cell viability assay

Cardiomyocytes cultured in 96-well plates were pre-incubated with losartan and simvastatin at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M for 1 h. Ang II (10^{-7} M) was added and incubated with the cells for a further 44 h. MTT was added to each well under sterile conditions (final concentration 5 g/l), and the plates were incubated for 4 h at 37°C. Untransformed MTT was removed by aspiration, and formazan crystals were dissolved in DMSO (150 μl per well). Formazan was quantified spectroscopically at 540 nm using an automated plate reader (Bio-Rad EIA Analyzer, Hercules, CA, US).

Analysis of apoptotic nuclei

After pre-treatment with losartan or simvastatin (both 10^{-5} M) or both together for 1 h, Ang II (10^{-7} M) was added and incubated with the cells for an additional 48 h. Cultured cells on coverslips (24-well plate) were washed three times with phosphate-buffered saline (PBS). To detect the characteristic features of apoptotic nuclei, fixed cardiomyocytes were stained with 0.5 ml Hoechst 33258, a fluorescent DNA-binding dye, for 5 min. Fluorescence (excited at 350 nm and emitted at 460 nm) was captured with a charge-coupled device camera under a fluorescent microscope. Apoptotic cells were identified by their typical morphological appearance, with chromatin condensation and nuclear fragmentation.

Analysis of cardiomyocyte apoptosis

The apoptotic ratios of cardiomyocytes in different groups were measured by flow cytometry according to the manufacturer's protocol. Double staining for FITC–Annexin V binding and for cellular DNA using propidium iodide was performed as follows. Following drug treatment, the medium was aspirated and cells were detached by trypsin. After washing twice with PBS, 1×10^6 cells were resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). FITC–Annexin V was added to a final concentration of 1 μg/ml Annexin V. A 0.1 ml volume of propidium iodide (10 μg/ml in binding buffer) was added, resulting in a final concentration of 1 μg/ml cell suspension. The mixture was incubated for 15 min in the dark at room temperature. Cellular fluorescence was measured by flow cytometry using a FACS-SCAN apparatus (Becton-Dickinson, Franklin Lakes, NJ, US). The percentage of apoptotic cells was determined using Modfit LT software (Verity Software House, Topsham, ME, US).

Detection of Bax and Bcl-2 protein expression

After incubation for 48 h, cells in different groups were collected by trypsin and adjusted to 10^6 cells/ml. Cardiomyocytes were then resuspended in alcohol for another 24 h at 4°C. Cells were washed with PBS and propidium iodide-labelled Bcl-2 or FITC-labelled Bax antibodies were added to the cells at a final concentration of 1 μg per 1×10^6 cells. Flow cytometric analysis was performed after 30 min of dark reaction.

Probability sum test

To determine whether the combination was synergistic or additive, we used the probability sum test (q test).[27,28] This test comes from classic probability analysis, and has been proposed for evaluating synergism between drugs. In the present work, the combinative effects of two agents was evaluated. The formula used was $q = P_{A+B}/[(P_A + P_B) - (P_A \times P_B)]$, where A and B represent the two drugs and P (probability) is the percentage inhibition; P_{A+B} is the actual percentage inhibition, $[(P_A + P_B) - (P_A \times P_B)]$ is the expected response rate; $(P_A + P_B)$ indicates the sum of the probabilities when drug A and drug B are used alone; $(P_A \times P_B)$ is the probability of inhibition when the two drugs are used independently. A q value below 0.85 indicates that the combination is antagonistic; a q value above 1.15 indicates synergism; a q value between 0.85 and 1.15 indicates that the effects are additive.

Statistical analysis

All data are expressed as mean ± SD. One-way analysis of variance was used to assess differences between drug groups and various related control groups, followed by the least squares difference post-hoc multiple comparisons test. $P < 0.05$ was considered significant. Analysis was performed using SPSS software (Chicago, IL, US).

Results

Effects of losartan and simvastatin on cell viability

Using the established in-vitro model of cardiomyocyte apoptosis induced by Ang II (10^{-7} M), we investigated the effects of losartan or simvastatin, alone and in combination, on cell viability following Ang II treatment. Ang II significantly decreased cell viability, whereas co-culture with either losartan or simvastatin at concentrations of 10^{-8}–10^{-4} M significantly dose-dependently improved cell viability compared with the Ang II group (Figure 1). The effects of losartan and simvastatin alone and in combination on cell viability with Ang II treatment are shown in Table 1. The data indicate that all three treatments protect the cardiomyocytes from Ang II-induced damage. Furthermore, co-treatment with losartan and simvastatin had a significantly greater effect on cell viability than either drug alone.

To exclude the possibility that either losartan or simvastatin decreased cardiomyocyte viability, we checked the effects of the two drugs separately and in combination

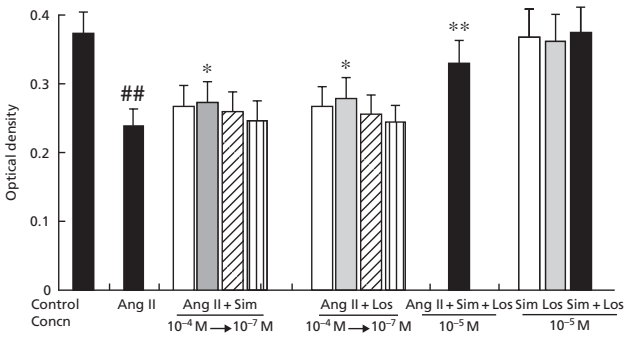

Figure 1 Effects of losartan (Los) and simvastatin (Sim), alone and in combination, on cardiomyocyte cell viability with and without angiotensin (Ang II) treatment. Values are means ± SD ($n = 3$ independent experiments). $^{\#\#}P < 0.01$ vs control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs Ang II group.

Table 1 Results of the probability sum test for cell viability and apoptosis in cardiomyocytes treated with angiotensin II (Ang II; 10^{-7} M) and losartan or simvastatin (both 10^{-5} M) alone and in combination

Parameter	Mean	*P* value (%)	*q* value
Cell viability (optical density)			
Ang II	0.238	–	
Ang II + simvastatin	0.273	14.71	
Ang II + losartan	0.279	17.23	
Ang II + losartan + simvastatin	0.330	30.25	1.31
Apoptosis (%)			
Ang II	21.98	–	
Ang II + simvastatin	15.82	28.03	
Ang II + losartan	16.10	26.75	
Ang II + losartan + simvastatin	9.42	57.14	1.21

P (probability) is the percentage of inhibition in each group; $q > 1.15$ indicates synergism. Values are from six experiments.

at a concentration of 10^{-5} M on cell viability without Ang II treatment (Figure 1). The results showed that neither losartan nor simvastatin affected cell viability.

Morphological characterisation of Ang II-induced cardiomyocyte apoptosis

Nuclear morphological changes were observed by Hoechst 33258 staining. As shown in Figure 2, visual inspection of Ang II-treated myocytes by fluorescence microscopy demonstrated that myocytes displayed morphological features characteristic of cell apoptosis. Compared with control cells, cells incubated with Ang II for 48 h became shrunken and retracted, and had condensed cytoplasm. In addition, Ang II-treated myocytes had either significantly more condensed chromatin or fragmented nuclei compared with vehicle-treated cells (Figure 2).

Protective effects of losartan and simvastatin against Ang II-induced apoptosis

To elucidate the protective nature of losartan and simvastatin on Ang II-induced apoptosis, we examined cell apoptosis using microscopy and flow cytometry. Cells were exposed to either losartan or simvastatin at concentrations of 10^{-8}–10^{-4} M for 48 h and subsequently stained with Hoechst 33258. The morphology of fragmented nuclei in apoptotic cells is displayed in Figure 2. These observations were confirmed by flow cytometry. We found that the percentage of apoptotic cells was markedly increased in the Ang II group compared with the control group. Compared with the Ang II group, the numbers of apoptotic cells were significantly decreased when co-cultured with either losartan or simvastatin, and this effect was markedly greater when cells were cultured with both losartan and simvastatin (Figure 3).

Synergistic interaction between losartan and simvastatin on cell viability and apoptosis

To study the nature of the interaction between losartan and simvastatin, their concentration-dependent inhibitory effects against Ang II-induced cardiomyocyte apoptosis were determined when the agents were used individually and in combination. A probability testing model was used to investigate the nature of the interaction between losartan and simvastatin, as described above. The q values were 1.31 for cell viability and 1.21 for cardiomyocyte apoptosis. These results suggested that the inhibitory effect of the combination of the two agents on apoptosis was larger than the expected additive effect with each drug alone, at both low and high doses (Figure 1, Table 1).

Effects of losartan and simvastatin on Bax and Bcl-2 protein expression

Bax proteins are pro-apoptotic whereas Bcl-2 proteins have anti-apoptotic actions. The ratio between Bcl-2 and Bax expression has been proposed as an important marker of myocardial cell survival probability. Flow cytometry analyses were conducted to determine whether the anti-apoptotic effects of losartan and simvastatin were due to a change in the ratio between Bcl-2 and Bax. Ang II induced a significant increase in Bax protein and a significant decrease in Bcl-2 protein expression in cardiomyocytes. Losartan or its

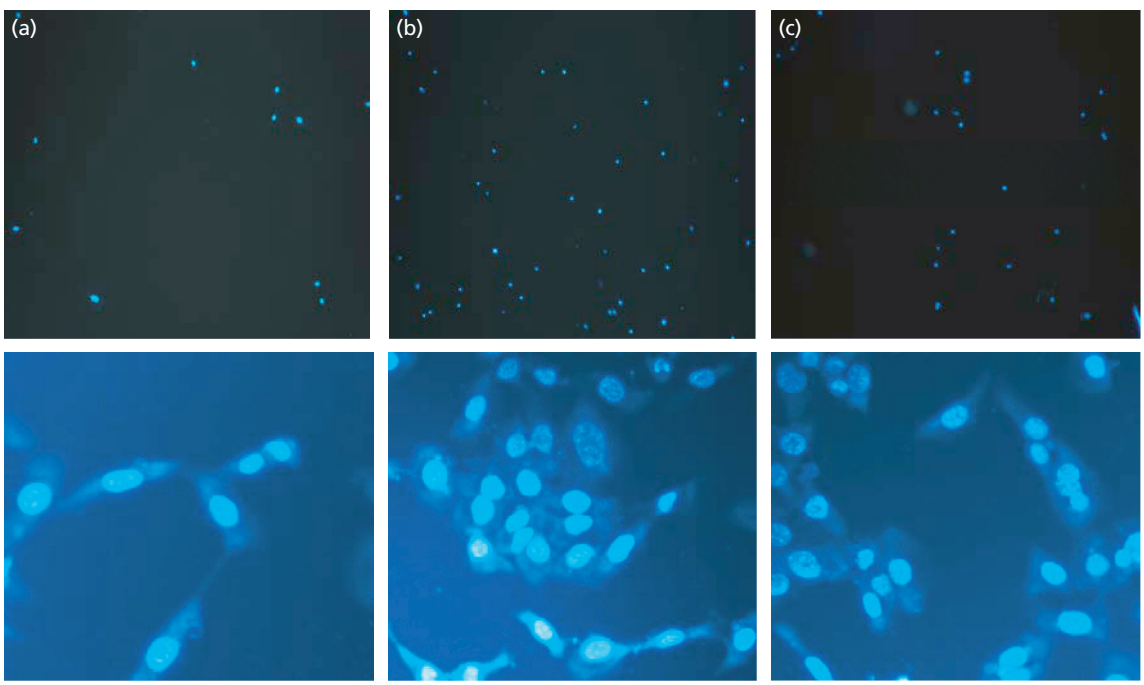

Figure 2 Morphological characterisation of cardiomyocyte apoptosis. Nuclear morphological changes were observed by Hoechst 33258 staining. Apoptotic cells were identified by their typical morphological appearance, with chromatin condensation and nuclear fragmentation. (a) Control; (b) angiotensin II (10^{-7} M); (c) angiotension II (10^{-7} M) + losartan and simvastatin (both 10^{-5} M). The upper row of images are at ×40 magnification and the lower row at ×200.

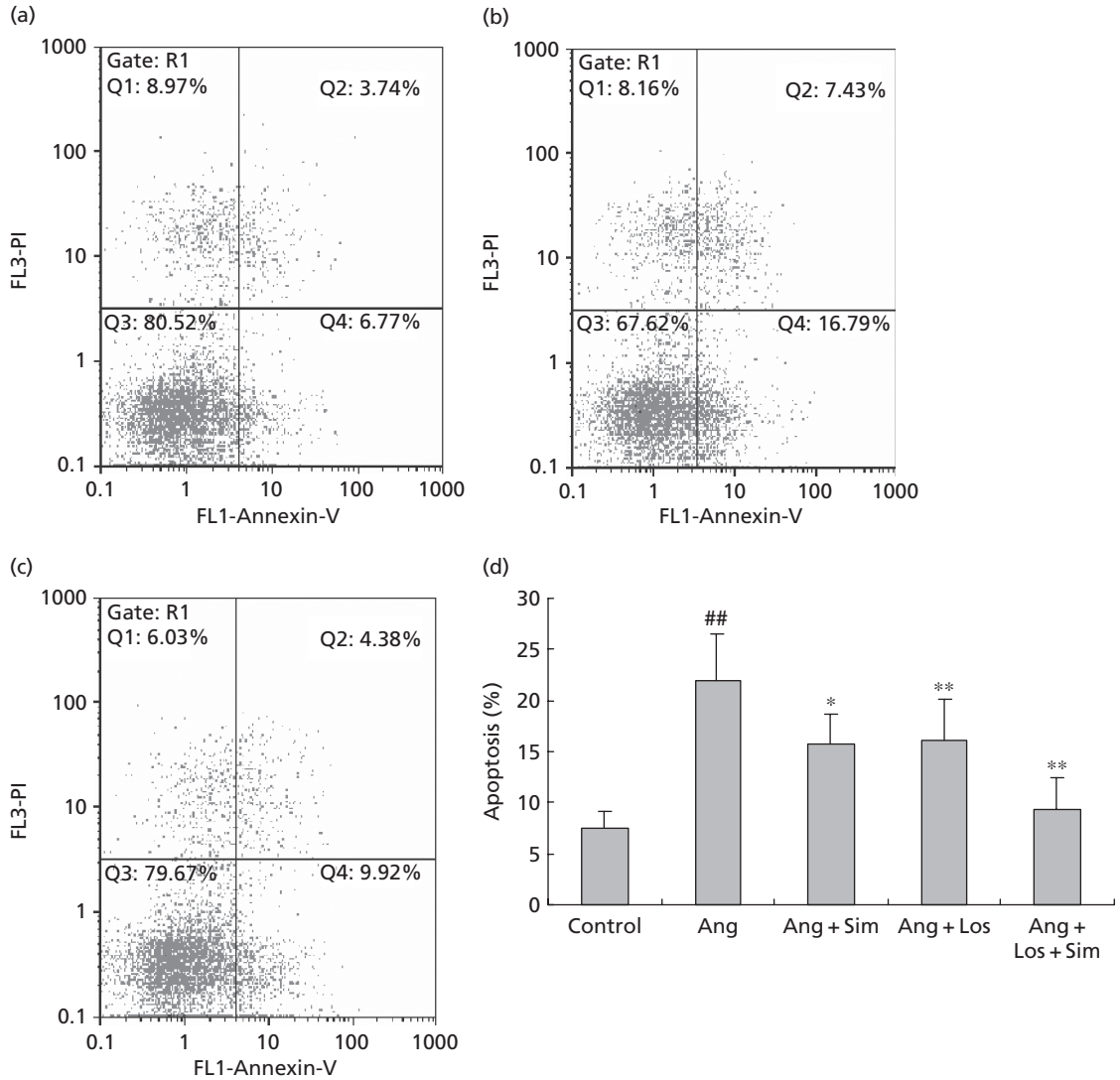

Figure 3 Effects of losartan (Los) and simvastatin (Sim) on cardiomyocyte apoptosis. The apoptotic ratio of cardiomyocytes in different groups were measured by flow cytometry using FITC-Annexin V/propidium iodide double staining. (a) Control; (b) angiotensin II (Ang; 10^{-7} M); (c) Ang (10^{-7} M) + Los and Sim (both 10^{-5} M). Values are means ± SD ($n = 6$). $^{\#\#}P < 0.01$ vs control group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Ang II group.

combination with simvastatin effectively prevented the Ang II-induced increase in the expression of Bax. However, treatment with simvastatin had no such effect (Figures 3–5).

Discussion

Losartan and simvastatin are two classic drugs widely used in the treatment of cardiovascular diseases, such as myocardial ischaemia, myocardial infarction and heart failure. The two drugs belong to two different classes, and their mechanisms of action are quite different. This study showed that apoptosis induced in cardiomyocytes by Ang II can be considerably reduced by the combination of losartan and simvastatin. We also demonstrated that losartan/simvastatin combination therapy had anti-apoptotic effects, and the effects of the two drugs were synergistic rather than additive. Ang II induced a significant increase in Bax protein expression but decreased expression of Bcl-2. Treatment with losartan in combination with simvastatin blocked the increased Bax expression and increased Bcl-2 expression, while simvastatin had no such effect.

Other mechanisms for the observed effect could involve a causal relationship with the AT_1 receptor. The interaction of the receptor and the action of these drugs needs further investigation.

Given that Ang II acts as an efficient inducer of apoptosis in adult and neonatal cardiomyocytes,[29,30] we used this model to investigate the synergism between losartan and simvastatin on cardiomyocyte apoptosis. When cardiomyocytes were exposed to Ang II, cell viability was significantly decreased and marked apoptosis was observed. Because a single assay for apoptosis is difficult to interpret, we assessed cardiomyocyte death using three separate assays. Morphological assessment of cardiomyocytes demonstrated that cell shrinkage, characteristic of

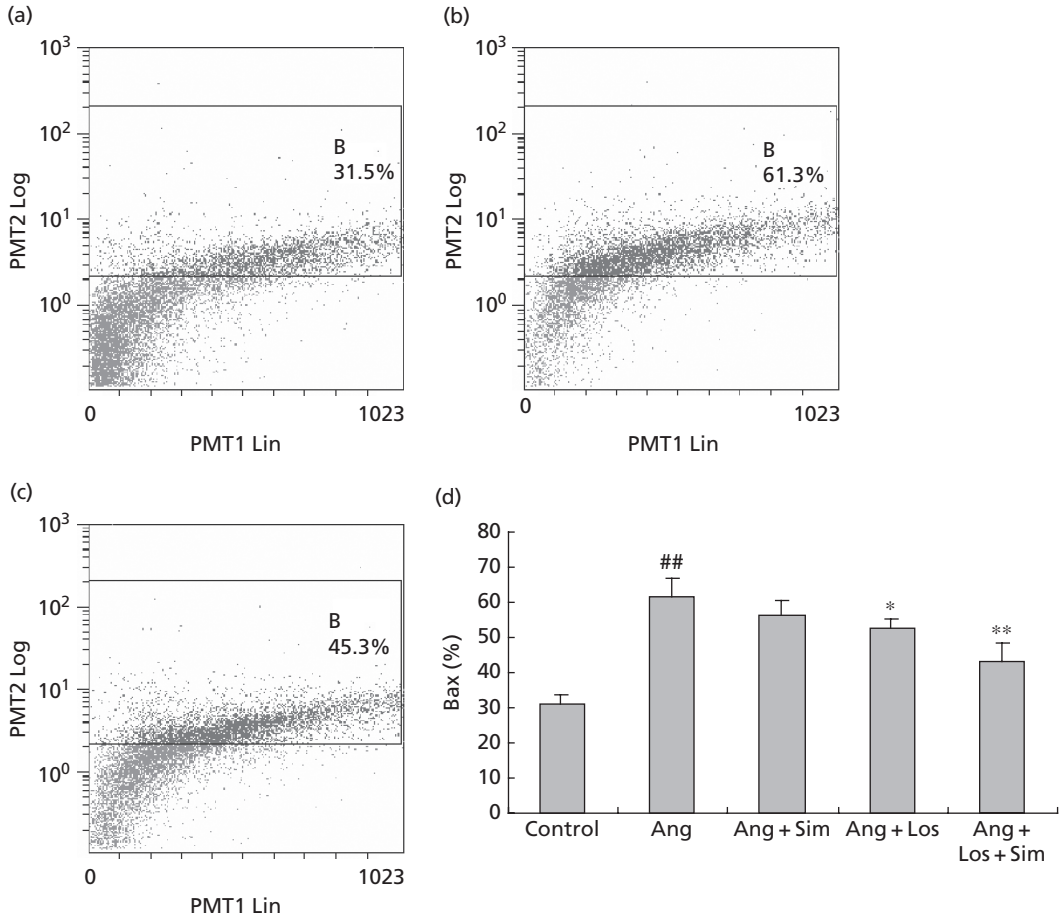

Figure 4 Effects of losartan (Los) and simvastatin (Sim) on Bax protein expression in cardiomyocytes. (a) Control; (b) angiotensin II (Ang; 10^{-7} M); (c) Ang (10^{-7} M) + Los and Sim (both 10^{-5} M). Values are means ± SD ($n = 6$). $^{\#\#}P < 0.01$ vs control group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Ang II group.

apoptosis, was more common in cells treated with Ang II compared with vehicle-treated cells. Flow cytometry analysis showed that the number of apoptotic cells was markedly increased in the Ang II-treated cells but decreased in the cells treated with losartan or simvastatin; this effect was exacerbated when the drugs were used in combination. Furthermore, the MTT assay demonstrated that cell viability in the losartan- and simvastatin-treated and vehicle-treated groups was much higher than that in cells treated with Ang II alone.

The probability sum test was used to determine whether the effect of the combination of the two drugs was synergistic. This test was first proposed by Jin in 1980[31] and subsequently adapted for evaluating the synergism of two antihypertensive drugs.[32,33] In the present work, we found that in cultured neonatal rat cardiomyocytes, cell viability and cardiomyocyte apoptosis was markedly influenced by pretreatment with either losartan or simvastatin, and that these effects were markedly enhanced when the two drugs were used in combination. The q values for cell viability and cardiomyocyte apoptosis were 1.31 and 1.21, respectively. Taken together, these findings clearly demonstrate that the treatment with losartan and simvastatin in combination has an overt synergistic effect on cardiomyocyte apoptosis induced by Ang II. In another study, we have also observed that chronic treatment with losartan and simvastatin protected cardiomyocytes from apoptotic death, and improved cardiac dysfunction and left ventricular remodelling, and attenuated the development of cardiac cachexia in a rat model of pressure overload-induced heart failure (unpublished data). We also found that losartan and simvastatin had cooperative effects (unpublished data). These studies suggest that the anti-apoptotic effects of losartan and simvastatin may be an important underlying mechanism accounting for their cardioprotective actions.

Cell death is controlled in part by a complex interplay between regulatory proteins such as pro-apoptotic and anti-apoptotic mediators, Bax and Bcl-2, respectively. In cardiomyocytes, Bcl-2 is a prototype for an anti-death or survival factor, whereas Bax accelerates the apoptotic process. The ratio of Bax to Bcl-2 determines death or survival of cardiomyocytes after an apoptotic stimulus.[34,35] We used flow cytometry analysis to detect Bax and Bcl-2 protein expression in cultured neonatal cardiomyocytes. Our data show that Ang II induced a significant decrease in the expression of Bcl-2 and a significant increase in Bax protein in cardiomyocytes compared with the control group. Losartan alone and in combination with simvastatin effectively upregulated Bcl-2 protein and down-regulated Bax protein expression, while simvastatin had no such effect. These observations suggest that losartan, alone or in

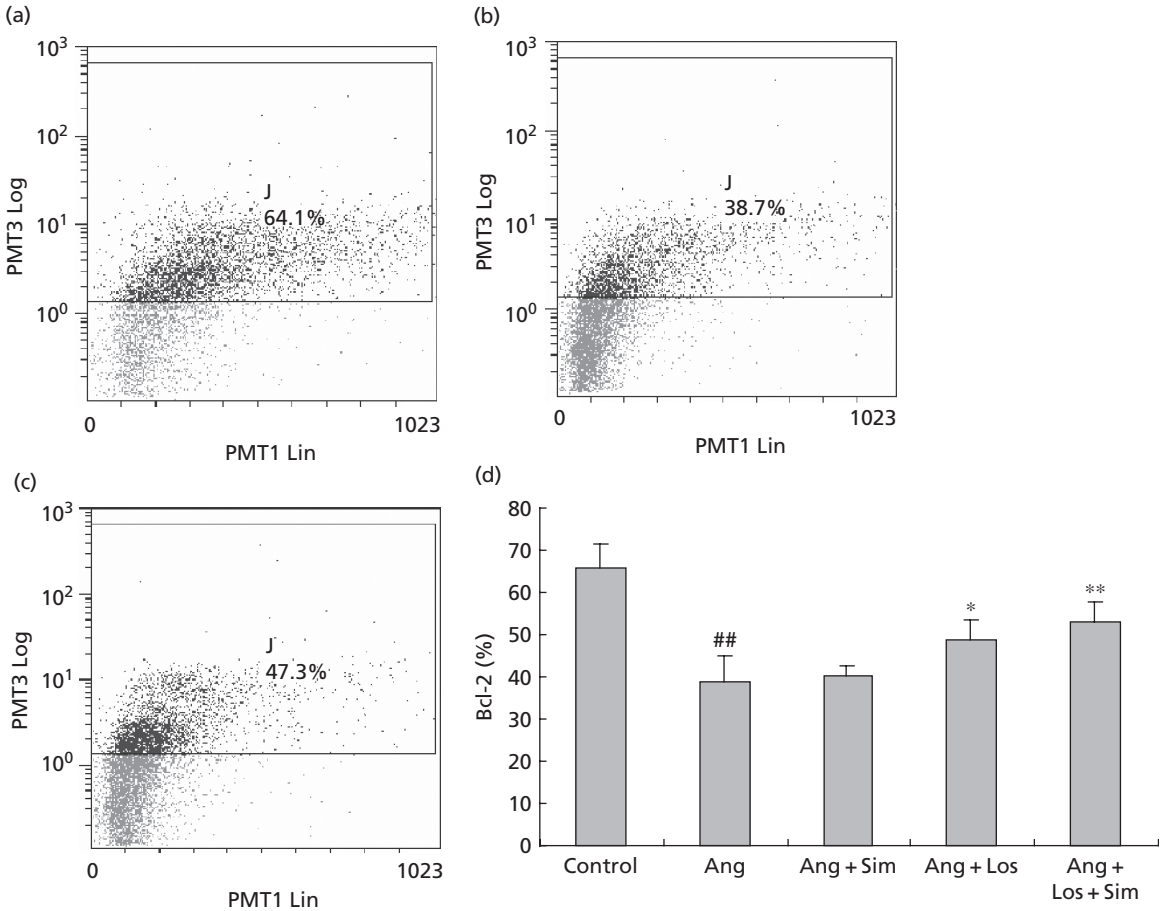

Figure 5 Effects of losartan (Los) and simvastatin (Sim) on Bcl-2 protein expression in cardiomyocytes. (a) Control; (b) angiotensin II (Ang; 10^{-7} M); (c) Ang (10^{-7} M) + Los and Sim (both 10^{-5} M). Values are means ± SD ($n = 6$). $^{\#\#}P < 0.01$ vs control group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Ang II group.

combination with simvastatin, modifies the imbalance of Bax and Bcl-2 in apoptotic cardiac cells.

Conclusions

Collectively the data demonstrate that the combination of losartan and simvastatin have an obviously synergistic effect on preventing apoptosis induced by Ang II in cultured neonatal rat cardiomyocytes. In addition, the mechanisms by which losartan and simvastatin protect cardiomyocytes from apoptosis are dissimilar. The anti-apoptotic mechanisms of losartan and simvastatin, and their combination, deserve further investigation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Acknowledgements

We thank Dr Hua Wang and Dr Andrew M. Miller for helpful discussions and careful reading of the manuscript.

References

1. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007; 35: 495–516.
2. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med* 2005; 258: 479–517.
3. Li Z *et al*. Increased cardiomyocyte apoptosis during the transition to heart failure in the spontaneously hypertensive rat. *Am J Physiol* 1997; 272: H2313–2319.
4. Wencker D *et al*. A mechanistic role for cardiac myocyte apoptosis in heart failure. *J Clin Invest* 2003; 111: 1497–1504.
5. Abbate A *et al*. Pathophysiologic role of myocardial apoptosis in post-infarction left ventricular remodeling. *J Am Coll Cardiol* 2002; 193: 145–153.
6. Li PF *et al*. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by bcl-2. *EMBO J* 1999; 21: 6027–6036.
7. Condorelli G *et al*. Increased cardiomyocyte apoptosis and changes in proapoptotic and antiapoptotic genes bax and bcl-2

during left ventricular adaptations to chronic pressure overload in the rat. *Circulation* 1999; 99: 3071–3078.
8. Xu J *et al*. Role of cardiac overexpression of Ang II in the regulation of cardiac function and remodeling postmyocardial infarction. *Am J Physiol Heart Circ Physiol* 2007; 293: H1900–1907.
9. Li HL *et al*. Overexpression of myofibrillogenesis regulator-1 aggravates cardiac hypertrophy induced by angiotensin II in mice. *Hypertension* 2007; 49: 1399–1408.
10. Ono H, Ishimitsu T Effects of angiotensin II type 1 and type 2 receptor on apoptosis. *Nippon Rinsho* 2002; 60: 1887–1892.
11. Yamada T *et al*. Angiotensin II type 2 receptor mediates programmed cell death. *Proc Natl Acad Sci USA* 1996; 93: 156–160.
12. Kajstura J *et al*. Angiotensin II induces apoptosis of adult ventricular myocytes in vitro. *J Mol Cell Cardiol* 1997; 29: 859–870.
13. Zhu YC *et al*. Role of angiotensin AT1 and AT2 receptors in cardiac hypertrophy and cardiac remodelling. *Clin Exp Pharmacol Physiol* 2003; 30: 911–918.
14. Simonis G *et al*. A novel activation process of protein kinase C in the remote, non-ischemic area of an infarcted heart is mediated by angiotensin-AT1 receptors. *J Mol Cell Cardiol* 2003; 35: 1349–1358.
15. Anavekar NS, Solomon SD. Angiotensin II receptor blockade and ventricular remodelling. *J Renin Angiotensin Aldosterone Syst* 2005; 6: 43–48.
16. Shimizu T *et al*. Long-term combined therapy with an angiotensin type I receptor blocker and an angiotensin converting enzyme inhibitor prolongs survival in dilated cardiomyopathy. *Jpn Heart J* 2002; 43: 531–543.
17. Suzuki G *et al*. Effects of the AT1 receptor antagonist eprosartan on the progression of left ventricular dysfunction in dogs with heart failure. *Br J Pharmacol* 2003; 138: 301–309.
18. Bauersachs J *et al*. Improvement of left ventricular remodeling and function by hydroxymethylglutaryl coenzyme A reductase inhibition with cerivastatin in rats with heart failure after myocardial infarction. *Circulation* 2001; 104: 982–985.
19. Hognestad A *et al.*, OPTIMAAL Investigators. Effect of combined statin and beta-blocker treatment on one-year morbidity and mortality after acute myocardial infarction associated with heart failure. *Am J Cardiol* 2004; 93: 603–606.
20. Landmesser U *et al*. Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase. *Circulation* 2004; 110: 1933–1939.
21. Dickstein K, Kjekshus J, the OPTIMAAL Steering Committee of the OPTIMAAL Study Group. Effects of losartan and captopril on mortality and morbidity in high-risk patients after acute myocardial infarction: the OPTIMAAL randomised trial: Optimal Trial in Myocardial Infarction with Angiotensin II Antagonist Losartan. *Lancet* 2002; 360: 752–760.
22. Laufs U *et al*. Impact of HMG CoA reductase inhibition on small GTPases in the heart. *Cardiovasc Res* 2002; 53: 911–920.
23. Maack C *et al*. Oxygen free-radical release in human failing myocardium is associated with increased activity of rac1-GTPase and represents a target for statin treatment. *Circulation* 2003; 108: 1567–1574.
24. Nickenig G *et al*. Statin-sensitive dysregulated AT1 receptor function and density in hypercholesterolemic men. *Circulation* 1999; 100: 2131–2134.
25. Tang SC *et al*. Angiotensin converting enzyme inhibitor but not angiotensin receptor blockade or statin ameliorates murine adriamycin nephropathy. *Kidney Int* 2008; 73: 288–299.
26. Goldenberg I *et al*. Adenosine protects against angiotensin II-induced apoptosis in rat cardiocyte cultures. *Mol Cell Biochem* 2003; 252: 133–139.
27. Xie HH *et al*. Synergism of atenolol and nitrendipine on hemodynamic amelioration and organ protection in hypertensive rats. *J Hypertens* 2005; 23: 193–201.
28. Wang J *et al*. Effects of nine antihypertensive drugs on blood pressure variability in sinoaortic-denervated rats. *Acta Pharmacol Sin* 2006; 27: 1013–1017.
29. Pang JJ *et al*. Hexarelin protects rat cardiomyocytes from angiotensin II-induced apoptosis in vitro. *Am J Physiol Heart Circ Physiol* 2004; 286: H1063–1069.
30. Mel'nikova NP *et al*. Angiotensin-II activates apoptosis, proliferation and protein synthesis in the left heart ventricle of newborn albino rats. *Int J Cardiol* 2006; 112: 219–222.
31. Jin ZJ. Addition in drug combination. *Acta Pharmacol Sin* 1980; 1: 70–76.
32. Han P *et al*. Synergism of hydrochlorothiazide and nitrendipine on reduction of blood pressure and blood pressure variability in spontaneously hypertensive rats. *Acta Pharmacol Sin* 2006; 27: 1575–1579.
33. Ling G *et al*. Effects of combination therapy with atenolol and amlodipine on blood pressure control and stroke prevention in stroke-prone spontaneously hypertensive rats. *Acta Pharmacol Sin* 2007; 28: 1755–1760.
34. Wassmann S *et al*. Inhibition of geranylgeranylation reduces angiotensin II-mediated free radical production in vascular smooth muscle cells: involvement of angiotensin AT1 receptor expression and Rac1 GTPase. *Mol Pharmacol* 2001; 59: 646–654.
35. Luo JD *et al*. Simvastatin inhibits cardiac hypertrophy and angiotensin-converting enzyme activity in rats with aortic stenosis. *Clin Exp Pharmacol Physiol* 1999; 26: 903–908.