

Lentiviral-mediated p38 MAPK RNAi attenuates aldosterone-induced myocyte apoptosis

YAN ZHOU^{1*}, YUANHONG LIANG^{2*}, JIE WEI¹, JING CHEN¹ and QIZHU TANG³

¹Department of Emergency, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060; ²Department of Cardiology, Guangdong General Hospital, Guangdong Cardiovascular Institute, Guangzhou, Guangdong 510080; ³Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Abstract. Aldosterone-induced myocyte apoptosis is an important component of cardiovascular disease. While the p38 mitogen-activated protein kinase (p38 MAPK) pathway has been shown to be crucial in myocyte apoptosis, whether aldosterone induces myocyte apoptosis through this pathway remains unclear. In the present study, three individual strands of p38 MAPK short hairpin RNA (ShRNA), delivered by lentiviral vectors (PGLV), were constructed and used to explore the role of p38 MAPK pathway activation in aldosterone-mediated myocyte apoptosis in cultured myocytes and normotensive rats. Aldosterone stimulation increased myocyte apoptosis, caspase-3 expression levels and p38 MAPK mRNA and protein expression levels *in vitro* and *in vivo*. PGLV-ShRNA3 transduction decreased aldosterone-mediated myocyte apoptosis and p38 MAPK mRNA and protein expression levels *in vitro* (all $P < 0.01$). PGLV-ShRNA3 transduction significantly decreased aldosterone-mediated myocyte apoptosis, p38 MAPK mRNA and protein expression levels in normotensive rats ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively). Results from the present study suggest that aldosterone directly induces myocyte apoptosis through the p38 MAPK pathway and the

gene silencing of p38 MAPK may protect cardiac myocytes from aldosterone-mediated apoptosis.

Introduction

Experimental and clinical evidence has confirmed that aldosterone induces deleterious structural and functional changes in the heart (1-3). Clinical trials have demonstrated that aldosterone antagonists improve the outcome of patients with heart failure and left ventricular dysfunction after myocardial infarction (MI) (4-6). Potential mechanisms for the contribution of aldosterone in cardiovascular diseases may include the induction of myocyte apoptosis and cardiovascular extracellular matrix turnover, sympathoadrenergic stimulation, endothelial dysfunction and/or myocardial structural and electrical remodeling (7,8).

Additionally, previous studies provide evidence that aldosterone-mediated myocyte apoptosis is an important component of cardiovascular diseases (9,10). Several mechanisms which may link aldosterone and apoptosis have been suggested, including the activation of the membrane receptor-mediated calcineurin-Bad, c-Jun N-terminal kinase (JNK) and ERK1/2 pathways (11-13). However, whether the mechanism of aldosterone-mediated apoptosis in cardiac myocytes involves the p38 mitogen-activated protein kinase (p38 MAPK) pathway remains controversial.

The p38 MAPK pathway is important in numerous cell processes, including myocyte apoptosis, hypertrophy and inflammation, which may contribute to progressive left ventricular remodeling post-MI and the transition to heart failure (14,15). Pharmacological inhibitors of p38 MAPK have been shown to decrease myocyte apoptosis and improve cardiac function and heart failure *in vitro* and *in vivo* (16,17). However, a number of studies have demonstrated that SB203580, a p38 MAPK inhibitor, has minimal effects on aldosterone-induced apoptosis (18). Thus, whether the inhibition of p38 MAPK improves aldosterone-mediated apoptosis requires clarification.

The present study aimed to provide further understanding of the mechanism of the association between aldosterone and apoptosis. We assessed the direct effects of gene silencing of the p38 MAPK signaling cascade to determine whether this

Correspondence to: Professor Qizhu Tang, Department of Cardiology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan, Hubei 430060, P.R. China
E-mail: qztang@whu.edu.cn

*Contributed equally

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular regulated protein kinases; GFP, green fluorescent protein; IFU, infectious unit; JNKs, Jun N-terminal kinases; MI, myocardial infarction; p38 MAPK, p38 mitogen-activated protein kinase; ShRNA, short hairpin RNA; TU, transducing units; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling

Key words: aldosterone, p38 mitogen-activated protein kinase, apoptosis, short hairpin RNA, lentiviral vector

was able to attenuate aldosterone-mediated myocyte apoptosis. We also explored a potential mechanism that may underlie these effects.

Materials and methods

Neonatal rat cardiac cell cultures. Cardiac cells were isolated from 1 to 3-day-old neonatal Sprague-Dawley rats and cultured, as described previously (19). Myocytes were seeded in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% horse serum and then transferred after 24 h to a defined medium, which prevented the growth of proliferating cells.

Design and cloning of lentiviral short hairpin RNA (ShRNA) vectors. The target ShRNAs against *Rattus norvegicus* mRNA for p38 MAPK (GenBank GI no. 157890411; Genepharma Co., Ltd., Shanghai, China) were as follows: ShRNA1, 5'-GGACCTCCTTATAGACGAATG-3'; ShRNA2, 5'-GAAGCTGTCGAGACCGTTTCA-3'; ShRNA3, 5'-GCCGAAGATGAACTTCGCAAA-3'; and the ShRNA control sequence, 5'-GCGAGGGCTGAAGTATATACA-3'. These ShRNAs were cloned into lentiviral vectors (PGLV; Genepharma Co., Ltd.) and a negative control PGLV was also designed (PGLV-NC). The lentiviral vectors used in this study were third generation, self-inactivating vectors that contained green fluorescent protein (GFP) (20). HEK-293T cells were co-transfected with appropriate amounts of the vector plasmids; helper construct, envelope plasmid and PGLVs containing ShRNA. The culture medium was collected over 48 h, concentrated by ultracentrifugation, aliquoted and stored at -80°C until it was used. The virus titer was calculated as the number of cells expressing GFP multiplied by the corresponding dilution and the titer of lentivirus was determined by a hole-by-dilution titer assay. The final titer of recombinant virus was 1×10^8 transducing units (TU)/ml. All constructs were verified by sequence analysis and results of the DNA sequencing were as expected.

Transduction of cultured myocytes and GFP fluorescence observation. Dissociated myocytes were infected with lentivirus vectors for 72 h. GFP fluorescence in the cells was monitored using a fluorescence microscope (Leica, Solms, Germany) at 24, 48 and 72 h post-transduction. At 48 h after infection, the cells were assayed for expression of the transgenes.

Administration of aldosterone and PGLV-ShRNAs in vivo. Procedures involving animals and their care were conducted in accordance with the guidelines approved by the China Association of Laboratory Animal Care. Male adult normotensive Sprague-Dawley rats (body weight, 280-300 g) were obtained from the Animal Research Center of Zhongshan University (Zhongshan, China). The rats were treated once with a hydrodynamic tail vein injection containing 2×10^8 infectious units (IFU) of PGLV-ShRNA2, -ShRNA3 or -NC (n=6). At 24 h after the hydrodynamic tail vein injection, d-aldosterone (1 mg/kg; Sigma Chemicals, St. Louis, MO, USA) was administered by gavage for 24 h. The control group received vehicle only (5% ethanol; n=6 per group).

Myocyte apoptosis in vivo and in vitro. Cells were cultured in a normal growth medium for 72 h following infection. To evaluate the effects of p38 MAPK, myocytes were pre-treated with PGLV-ShRNA1, -ShRNA2, -ShRNA3 or -NC for 48 h and stimulated with 10^{-5} mol/l aldosterone for 24 h. Control myocytes were incubated in DMEM. Cultured rat myocyte apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL). Positive (myocardial sections treated with DNase I) and negative controls (omission of biotin-16-dUTP or TdT) were also included. Interference contrast was used to exclude apoptotic nuclei of a non-cardiomyocyte origin from cell counts.

Caspase-3 activity. Caspase-3 enzymatic activity in myocytes was determined using a CPP32 assay kit (MBL), which detects the production of the chromophore p-nitroanilide after it is cleaved from the peptide substrate DEVD p-nitroanilide, as described previously (21).

Real-time polymerase chain reaction (PCR) analysis. Total RNA from cultured and rat myocytes was extracted using an RNA isolation kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The reactions were carried out on a real-time PCR system (iQ5 real-time PCR; Bio-Rad, Hercules, CA, USA) under the following cycle conditions: 95°C for 15 sec, 45 cycles at 95°C for 5 sec and at 60°C for 30 sec. A standard curve for p38 MAPK was generated using serially diluted total RNA from myocytes and this was used to quantify relative p38 MAPK mRNA levels. The sequences of the p38 MAPK gene primers were as follows: forward, 5'-AAC CTGTCCCCGGTGGGCTCG-3'; and reverse, 5'-CGATGT CCCGTCTTTGTATGA-3'. GAPDH was used as a control for normalization. The primers of GAPDH were as follows: forward, 5'-GAAGGTGAAGGTCGGAGTC-3'; and reverse, 5'-GAAGATGGTGATGGGATTTC-3'. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen).

Western blot analysis. Cultured and rat myocyte protein extracts (20 µg) were resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. Membranes were incubated with a p38 MAPK antibody (1:2000 anti-p38 MAPK; Cell Signaling Technology, Inc., Danvers, MA, USA), a caspase-3 antibody (1:1000 anti-cleaved caspase-3; Cell Signaling Technology, Inc.) and β -actin (1:2000; Sigma). The membranes were then washed with TBS containing 0.05% Tween-20, incubated with an anti-mouse immunoglobulin G horseradish peroxidase secondary antibody (1:2000; Sigma) and washed again. Protein expression was analyzed using the BandsScan software and normalized by the quantity of β -actin on the same membrane.

Statistical analysis. Results are expressed as mean \pm SEM. Data sets were analyzed using one-way analysis of variance (Kruskal-Wallis analysis of variance by ranks) followed by Bonferroni's post-hoc test for comparisons between the groups. $P < 0.05$ was considered to indicate a statistically significant difference.

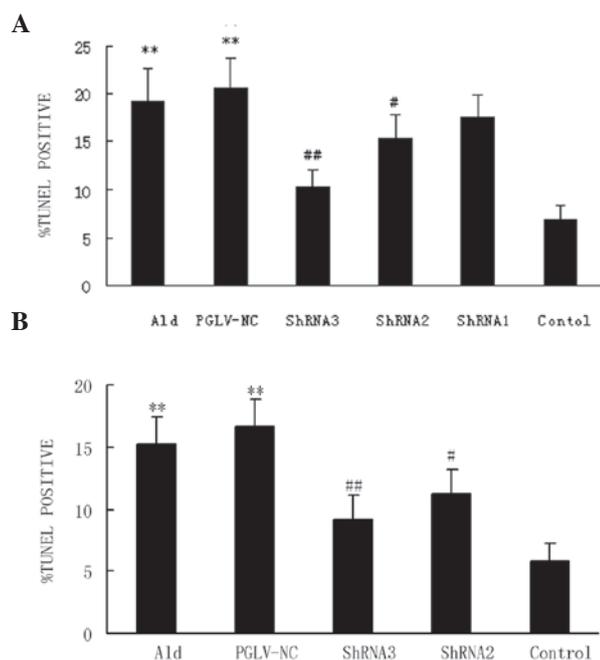


Figure 1. Myocyte apoptosis induced by aldosterone *in vitro* and *in vivo*. (A) A graph showing the quantification of apoptotic cells in six groups *in vitro* (n=6 per group). Myocytes were pre-treated with PGLV-ShRNA1, -ShRNA2, -ShRNA3 or -NC for 48 h and then stimulated with 10^{-5} mol/l aldosterone for 24 h. Control myocytes were incubated with DMEM. Groups were as follows: Ald, aldosterone group (myocytes were stimulated with aldosterone); PGLV-NC, negative control PGLV. *P<0.05 vs. the control group, #P<0.05 vs. the Ald group. (B) A graph showing the quantification of apoptotic cells in five groups *in vivo* (n=6 per group). At 24 h after the hydrodynamic tail vein injection, d-aldosterone (1 mg/kg) was administered by gavage for 24 h. Groups were as follows: Ald, aldosterone group; PGLV-NC, negative control PGLV; Control, tail vein injection contained vehicle only. *P<0.05 vs. the control group, #P<0.05 vs. the Ald group. PGLV, lentiviral vectors; ShRNA, short hairpin RNA; DMEM, Dulbecco's modified Eagle's medium.

Results

Lentiviral vector transduction in cultured cardiac myocytes.

The lentiviral vectors were transduced into cardiac cells *in vitro* for 72 h. No significant change in the cell morphology and no cell death were observed. The transduction efficiency was measured by the frequency of GFP-positive cells. A strong GFP signal was visible in cardiac cells 48 h after transduction with PGLV-ShRNA, indicating that the recombinant lentivirus led to the successful transduction of cardiac myocytes. As demonstrated by the representative experiment shown, >70% of cardiomyocytes were transduced successfully.

Myocyte apoptosis in vitro and in vivo. The stimulation of myocytes with 10^{-5} mol/l aldosterone significantly increased the level of myocyte apoptosis compared with the serum-deprived controls *in vitro* (19.31 ± 3.35 vs. $6.86 \pm 1.56\%$; P<0.01; Fig. 1A). PGLV-NC and PGLV-ShRNA1 had no significant effect on apoptosis compared with the aldosterone group *in vitro*. PGLV-ShRNA2 and -ShRNA3 decreased the level of myocyte apoptosis (to 10.2 and 15.4%, respectively) compared with the aldosterone group *in vitro* (P<0.05 and P<0.01, respectively; Fig. 1A). The gavage of d-aldosterone for 24 h significantly increased the level of myocyte apoptosis compared with the vehicle group in normotensive rats

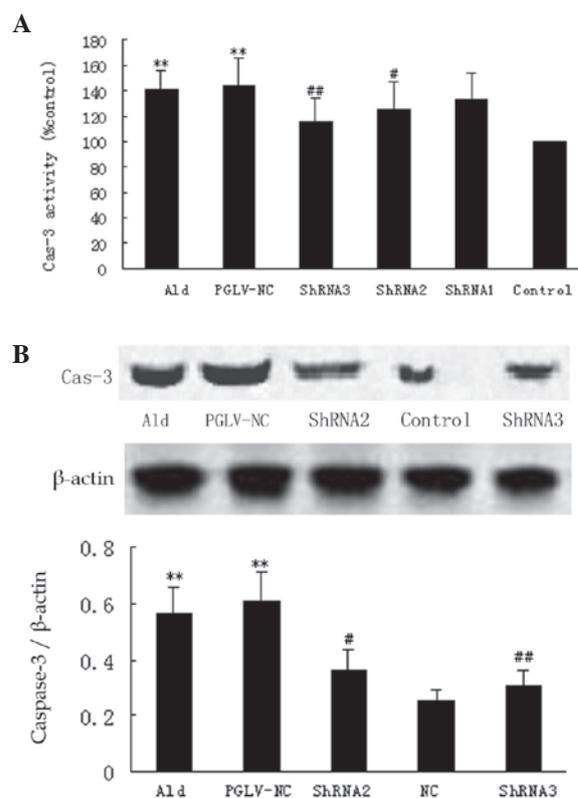


Figure 2. Caspase-3 expression *in vitro* and *in vivo*. (A) A graph showing the quantification of caspase-3 expression in six groups *in vitro* (n=6 per group). Myocytes were pre-treated with PGLV-ShRNA1, -ShRNA2, -ShRNA3 or -NC for 48 h and then stimulated with 10^{-5} mol/l aldosterone for 24 h. Control myocytes were incubated with DMEM. Groups were as follows: Ald, aldosterone group (myocytes were stimulated with aldosterone); PGLV-NC, negative control PGLV. *P<0.05 vs. the control group, #P<0.05 vs. the Ald group. (B) Western blotting and a graph showing western blot analysis of caspase-3 expression in five groups *in vivo* (n=6 per group). At 24 h after the hydrodynamic tail vein injection, d-aldosterone (1 mg/kg) was administered by gavage for 24 h. Groups were as follows: Ald, aldosterone group; PGLV-NC, negative control PGLV; Control, tail vein injection contained vehicle only. *P<0.05 vs. the control group, #P<0.05 vs. the Ald group. PGLV, lentiviral vectors; ShRNA, short hairpin RNA; DMEM, Dulbecco's modified Eagle's medium.

(15.20 ± 2.18 vs. $5.83 \pm 1.42\%$; P<0.01; Fig. 1B). PGLV-NC had no significant effect on apoptosis compared with the aldosterone group in normotensive rats (16.65 ± 2.24 vs. $15.20 \pm 2.18\%$; P>0.05). Pre-treatment with PGLV-ShRNA2 and -ShRNA3 significantly decreased the level of myocyte apoptosis (to 9.2 and 11.3%, respectively) compared with the aldosterone group *in vitro* (P<0.05 and P<0.01, respectively; Fig. 1B).

Caspase-3 expression in vitro and in vivo. The stimulation of myocytes with 10^{-5} mol/l aldosterone significantly increased the level of caspase-3 expression (to 41%) compared with the serum-deprived controls *in vitro* (P<0.01; Fig. 2A). PGLV-NC and -ShRNA1 had no significant effect on caspase-3 compared with the aldosterone group *in vitro*. PGLV-ShRNA2 and -ShRNA3 decreased the level of caspase-3 (to 10.6% and 17.7%, respectively) compared with the aldosterone group *in vitro* (P<0.05 and P<0.01, respectively; Fig. 2A). The gavage of d-aldosterone for 24 h significantly increased the level of caspase-3 expression compared with the vehicle group in normotensive rats (P<0.01; Fig. 2B). PGLV-NC had no

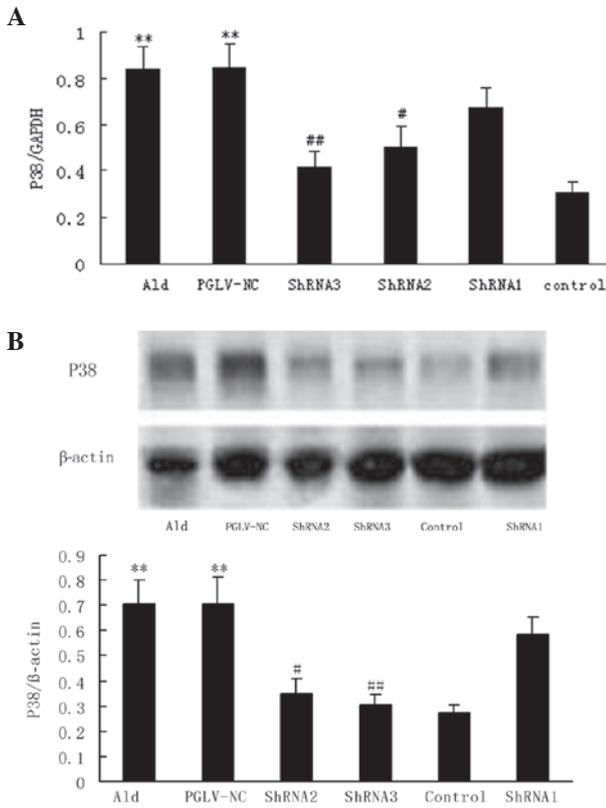


Figure 3. P38 MAPK expression *in vitro*. (A) A graph showing real-time PCR analysis of p38 MAPK mRNA levels in six groups *in vitro* (n=6 per group). (B) Western blotting and a graph showing western blot analysis of p38 MAPK protein levels in six groups *in vitro* (n=6 per group). Myocytes were pre-treated with PGLV-ShRNA1, -ShRNA2, -ShRNA3 or -NC for 48 h and then stimulated with 10⁻⁵ mol/l aldosterone for 24 h. Control myocytes were incubated with DMEM. Groups were as follows: Ald, aldosterone group (myocytes were stimulated with aldosterone); PGLV-NC, negative control PGLV. *P<0.05 vs. the control group, #P<0.05 vs. the Ald group. p38 MAPK, p38 mitogen-activated protein kinase; PCR, polymerase chain reaction; PGLV, lentiviral vectors; ShRNA, short hairpin RNA; DMEM, Dulbecco's modified Eagle's medium.

significant effect on caspase-3 expression compared with the aldosterone group in normotensive rats. Pre-treatment with PGLV-ShRNA2 and -ShRNA3 significantly decreased the level of caspase-3 expression (to 35.0% and 46.5%, respectively) compared with the aldosterone group *in vivo* (P<0.05 and P<0.01, respectively; Fig. 2B).

p38 MAPK expression in myocytes. With aldosterone treatment, P38 MAPK mRNA and protein expression levels were increased 2.7- and 2.6-fold, respectively, compared with the serum-deprived controls *in vitro* (P<0.01 and P<0.01, respectively; Fig. 3). PGLV-NC and -ShRNA1 had no significant effect on p38 MAPK mRNA or protein expression compared with the aldosterone group *in vitro*. PGLV-ShRNA2 transduction resulted in an ~39.9 and 49.9% reduction of p38 MAPK mRNA and protein expression levels, respectively, compared with the aldosterone group *in vitro* (P<0.05 and P<0.05, respectively). PGLV-ShRNA3 transduction resulted in an ~50.1 and 56.7% reduction of p38 MAPK mRNA and protein expression levels, respectively, compared with the aldosterone group *in vitro* (P<0.01 and P<0.01, respectively; Fig. 3).

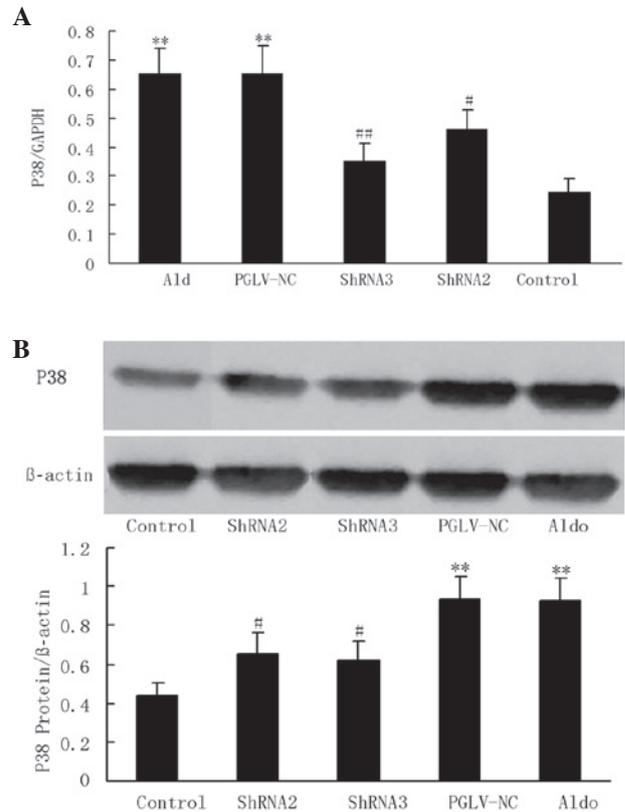


Figure 4. P38 MAPK expression in normotensive rats. (A) A graph showing real-time PCR analysis of p38 MAPK mRNA levels in five groups of normotensive rats (n=6 per group). (B) Western blotting and a graph showing western blot analysis of p38 MAPK protein levels in five groups of normotensive rats (n=6 per group). At 24 h after the hydrodynamic tail vein injection, d-aldosterone (1 mg/kg) was administered by gavage for 24 h. Groups were as follows: Ald, aldosterone group; PGLV-NC, negative control PGLV; Control, tail vein injection contained vehicle only. *P<0.05 vs. the control group, #P<0.05 vs. the Ald group. p38 MAPK, p38 mitogen-activated protein kinase; PCR, polymerase chain reaction; PGLV, lentiviral vectors; ShRNA, short hairpin RNA.

P38 MAPK expression in normotensive rats. With aldosterone treatment, P38 MAPK mRNA and protein expression levels were increased 2.2- and 2.6-fold, respectively, compared with the vehicle group in normotensive rats (P<0.01 and P<0.01, respectively; Fig. 4). PGLV-NC had no significant effects on p38 MAPK mRNA or protein expression compared with the aldosterone group *in vivo*. PGLV-ShRNA2 transduction resulted in an ~29.3 and 29.4% reduction in p38 MAPK mRNA and protein expression levels, respectively, compared with the aldosterone group *in vivo* (P<0.05 and P<0.05, respectively). PGLV-ShRNA3 transduction resulted in an ~46 and 33.6% reduction in p38 MAPK mRNA and protein expression levels, respectively, compared with the aldosterone group *in vivo* (P<0.01 and P<0.05, respectively; Fig. 4).

Discussion

The present study demonstrated the direct adverse effect of aldosterone on cardiomyocyte p38 MAPK expression and apoptosis. Previous studies have demonstrated that aldosterone directly induces myocyte apoptosis in a dose- and time-dependent manner (11,12). Our results showed that aldo-

sterone (10^{-5} mol/l for 24 h) significantly increased the level of myocyte apoptosis. A previous study also demonstrated that d-aldosterone (1 mg/kg, infused for 24 h) was able to stimulate cardiac myocyte apoptosis in adult normotensive rats (22,23). Our results showed that exposure to 10^{-5} mol/l aldosterone treatment for 24 h significantly increased myocyte apoptosis 2.8-fold compared with the serum-deprived control *in vitro*. The gavage of d-aldosterone for 24 h significantly increased myocyte apoptosis 2.6-fold compared with the vehicle group in normotensive rats.

Apoptosis is important in cardiovascular disease and may contribute to the development and progression of cardiac dysfunction and heart failure (8-10). *In vitro* and *in vivo* studies have suggested a number of potential mechanisms of these adverse effects, including the theory that aldosterone induces apoptosis in myocytes via activation of the calcineurin, p38 MAPK, JNK and ERK1/2 pathways. Ma *et al* (16) and See *et al* (17) reported that treatment with the p38 MAPK inhibitor SB203580 decreased myocyte apoptosis and improved postischemic cardiac function. The cardioprotective effects of SB203580 were shown to be closely associated with its ability to inhibit p38 MAPK. However, another study showed that SB203580 had minimal effects on aldosterone-mediated apoptosis (18). Of the several possible mechanisms which may underly the effects of aldosterone on cardiomyocyte apoptosis, our results suggest a key role for p38 MAPK activation that is known to be associated with cardiomyocyte apoptosis.

Despite observations that p38 MAPK inhibitors have a cardioprotective role in isolated ventricular myocytes and *in vivo*, p38 MAPK inhibitors may also affect other signaling pathways, including the JNK and ERK1/2 pathways. Whether p38 MAPK gene silencing ameliorates aldosterone-mediated cardiomyocyte apoptosis has rarely been investigated. Compared with gene knockout techniques, RNAi-based gene silencing is more rapid and cost-effective (24). Various delivery methods for the expression of ShRNA include direct application of naked siRNA and the use of lipid-based delivery vehicles. However, these methods are limited, due to low transduction efficiencies, weak control of gene expression and the short duration of effects (25). It is possible to achieve targeted ShRNA against p38 MAPK to cardiac myocytes by lentiviral vectors and this may be exploited as a novel approach to refine our understanding of the mechanisms of myocyte apoptosis.

In the present study, three individual strands of p38 MAPK ShRNA were constructed, delivered by lentiviral vectors, to investigate their effects on aldosterone-mediated myocyte apoptosis *in vitro* and *in vivo*. PGLV-ShRNA3 transduction significantly decreased the levels of myocyte apoptosis and caspase-3 expression, which were associated with significant reductions in p38 MAPK mRNA and protein expression levels. Thus, the PGLV-ShRNA3 sequence was a specific ShRNA target. The present study also demonstrated that p38 MAPK PGLV-ShRNA3 protected against aldosterone-mediated peri-infarct myocyte apoptosis and attenuated pathological cardiac remodeling and LV dysfunction in post-MI aldosterone overload rats (unpublished data). Based on these findings, we conclude that aldosterone induced cardiac dysfunction and apoptosis by activating p38 MAPK. Gene silencing of the p38 MAPK signaling cascade was able to attenuate aldosterone-mediated myocyte apoptosis.

Acknowledgements

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