

Darapladib, a Lipoprotein-Associated Phospholipase A2 Inhibitor, Reduces Rho Kinase Activity in Atherosclerosis

Juan Zhang^{1*}, Dong-Ling Xu^{1*}, Xiao-Bo Liu², Shao-jie Bi¹, Tong Zhao¹, Shu-Jian Sui¹, Xiao-Ping Ji³, and Qing-Hua Lu¹

¹Department of Cardiology, The Second Hospital of Shandong University, Jinan, Shandong Province;

²Shandong Blood Center, Jinan, Shandong Province;

³The Key Laboratory of Cardiovascular Remodeling and Function Research of the Chinese Ministry of Education and Public Health, Shandong University Qilu Hospital, Jinan, Shandong Province, China.

Purpose: Increased lipoprotein-associated phospholipase A2 (Lp-PLA2) activity and Rho kinase activity may be associated with atherosclerosis. The principal aim of this study was to examine whether darapladib (a selective Lp-PLA2 inhibitor) could reduce the elevated Lp-PLA2 and Rho kinase activity in atherosclerosis.

Materials and Methods: Studies were performed in male Sprague-Dawley rats. The atherosclerosis rats were prepared by feeding them with a high-cholesterol diet for 10 weeks. Low-dose darapladib (25 mg·kg⁻¹·d⁻¹) and high-dose darapladib (50 mg·kg⁻¹·d⁻¹) interventions were then administered over the course of 2 weeks.

Results: The serum levels of triglycerides, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), high-sensitivity C-reactive protein (hs-CRP), and Lp-PLA2, significantly increased in atherosclerosis model groups, as did Rho kinase activity and cardiomyocyte apoptosis ($p < 0.05$ vs. sham group), whereas nitric oxide (NO) production was reduced. Levels of TC, LDL-C, CRP, Lp-PLA2, and Rho kinase activity were respectively reduced in darapladib groups, whereas NO production was enhanced. When compared to the low-dose darapladib group, the reduction of the levels of TC, LDL-C, CRP, and Lp-PLA2 was more prominent in the high-dose darapladib group ($p < 0.05$), and the increase of NO production was more prominent ($p < 0.05$). Cardiomyocyte apoptosis of the high-dose darapladib group was also significantly reduced compared to the low-dose darapladib group ($p < 0.05$). However, there was no significant difference in Rho kinase activity between the low-dose darapladib group and the high-dose darapladib group ($p > 0.05$).

Conclusion: Darapladib, a Lp-PLA2 inhibitor, leads to cardiovascular protection that might be mediated by its inhibition of both Rho kinase and Lp-PLA2 in atherosclerosis.

Key Words: Atherosclerosis, lipoprotein-associated phospholipase A2 darapladib, Rho kinase, apoptosis

Received: March 9, 2015 Revised: July 9, 2015

Accepted: August 4, 2015

Co-corresponding authors: Dr. Juan Zhang, Department of Cardiology, The Second Hospital of Shandong University, Jinan 250033, Shandong Province, China. Tel: 86-85875089, Fax: 86-85875090, E-mail: awwa6940@sina.com and Dr. Qing-Hua Lu, Department of Cardiology, The Second Hospital of Shandong University, Jinan 250033, Shandong Province, China. Tel: 86-85875466, Fax: 86-85875090, E-mail: lqhzy@163.com

*Juan Zhang and Dong-Ling Xu contributed equally to this work.

•The authors have no financial conflicts of interest.

© Copyright: Yonsei University College of Medicine 2016

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

Atherosclerosis is the most common cause of cardiovascular diseases, such as unstable angina, myocardial infarction, stroke, and ischemic heart failure, and it is estimated that it will become the leading cause of death worldwide by the year 2020.¹ Atherosclerosis is the result of hyperlipidemia and lipid oxidation and is characterized by inflammatory reactions, endothelial dysfunction, smooth muscle cell proliferation, extracellular matrix alteration, thrombosis and apoptosis.

Rho kinase, one of the effectors of the small GTP-binding protein Rho, has been shown to play an important role in many major cardiovascular diseases, such as hypertension,^{2,3} heart

failure,⁴ myocardial infarction, ischemia/reperfusion injury,^{5,6} and atherosclerosis.⁷ Rho kinase consists of an N-terminal located kinase domain, followed by a long coiled-coil domain and a Rho-binding domain, and a C-terminal Pleckstrin-homology domain. Rho kinase activity can be regulated by several distinct mechanisms, such as the Rho/Rho-kinase pathway⁸ and the Caspase3/Rho-kinase pathway.⁹ Y-27632 and fasudil are non-isoform-selective Rho kinase inhibitors. Certain lipids, such as arachidonic acid, may effectively stimulate Rho kinase activity independent of RhoA binding.¹⁰ The lipids seem to bind to the regulatory C-terminus of Rho kinase, disrupting the autoinhibitory interaction and thus leading to kinase activation.¹¹ Furthermore, other negative regulators have been found that bind to and inhibit Rho kinase activity.

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a key enzyme responsible for degrading platelet-activating factor and oxidized-LDL (ox-LDL). Many experimental studies have shown that PLA2 is involved in lipid metabolism and immunoinflammatory response and participates in the development of atherosclerosis.^{12,13} Darapladib is an oral, investigational, highly potent, and selective Lp-PLA2 inhibitor.

The principal aim of this study was to examine whether darapladib could reduce the elevated Lp-PLA2 and Rho kinase activity in a dose-dependent manner in a rat atherosclerosis model.

MATERIALS AND METHODS

All procedures were performed in conformity with the Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Animal model and experimental protocol

Forty-eight male Sprague-Dawley rats (body weight 200–220 g, from Shandong University, Shandong Province, China) were maintained under conditions of standard lighting (alternating 12-h light/dark cycles), temperature (22±0.5°C), and humidity (60±10%) for at least 1 week before the experiments. The rats were then randomly divided into two groups. Twelve rats given a normal diet were designated as a sham group, and the other 36 rats were fed with a high-cholesterol diet for 10 weeks to induce atherosclerosis.^{14,15} The high-cholesterol diet contained 3% cholesterol, 0.5% cholic acid, 0.2% 6-propyl 2-thiouracil, 5% sucrose, and 3% lard. Subsequently, the 36 hyperlipidemic rats were randomly and evenly assigned to three groups: a control group that was administered normal saline, a low-dose darapladib group that received darapladib by gavage (25 mg·kg⁻¹·d⁻¹, reconstituted in normal saline), and a high-dose darapladib group that received darapladib by gavage (50 mg·kg⁻¹·d⁻¹, reconstituted in normal saline). The duration darapladib intervention was 2 weeks. At the end of the experiment, all rats were euthanized via injection of an overdose of sodium pentobarbital solution. The hearts were used for histological and biochemical studies.

Plasma lipid and biological analysis

Fasting blood was taken for laboratory examination before the beginning of the study, after 10 weeks of model production, and after 2 weeks of intervention. Blood samples were collected under chloral hydrate anesthesia and centrifuged at 3500 rpm for 15 minutes using a refrigerated centrifuge (HEMA, Zhuhai, China), and the serum was transferred into a separate vial and stored at 4°C. Serum concentrations of triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and high-sensitivity C-reactive protein (hs-CRP) were measured via enzymatic assays using an automated biochemical analyzer (Cobas 8000, Roche, Basel, Switzerland).

Plasma level of Lp-PLA2 was measured using a quantitative sandwich enzyme immunoassay (commercial ELISA kits) according to the manufacturer's recommendation (CoWin Bioscience Co., Ltd., Beijing, China). Additionally, serum level of nitric oxide (NO) was evaluated based on the nitrite reductase method using a Total Nitric Oxide Kit (Beyotime, Shanghai, China, S0023).

TUNEL staining

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique was used to evaluate apoptotic activity. Myocardial tissues were embedded in paraffin and cut into 5-mm thick slices. The sections were deparaffinized and rehydrated with serial changes of xylene and ethanol. Proteinase K (20 mg/L) was applied to each section for 30 min with the intention of producing optimal proteolysis. The endogenous peroxidase activity was inhibited with 3% hydrogen peroxide for 10 min. A commercial apoptosis detection kit (Roche, Basel, Switzerland) was used. The terminal deoxynucleotidyl transferase (TdT) reaction was carried out for 1 h at 37°C in a humidified chamber, and DAB chromogen was then applied. Hematoxylin was used as a counterstain. TUNEL-positive cells were calculated by randomly counting ten fields of the section and were expressed as a percentage of normal nuclei.¹⁶

Assay of protein concentration

The protein concentration of heart tissue was determined based on the method of Lowry with bovine serum albumin as the standard.¹⁷

Western blotting

Rho kinase activity was assessed by examining the phosphorylation state of myosin phosphatase targeting subunit 1 (MYPT-1), a well-established Rho kinase specific substrate. MYPT-1 and p-MYPT-1 levels were determined by western blot analysis. Equal amounts of protein (50 µg) were fractionated on 14% sodium dodecyl sulfonate (SDS)-polyacrylamide gels in running buffer [25 mM Tris, 192 mM glycine, 0.1% (wt/vol) SDS, pH 8.3] at 90 V and then electroblotted to nitrocellulose membranes. Membranes were blocked at room temperature with 5% nonfat

milk in Tris-buffered saline (25 mM Tris, 137 mM NaCl, and 2.7 mM KCl) containing 0.05% Tween-20 (TBS-T) and then incubated overnight at 4°C with the following primary antibodies: β-actin (Santa Cruz, TX, USA; dilutions: 1:1000; molecular weight of β-actin: 43 kD), rabbit polyclonal MYPT-1 antibody (Bioworld, MN, USA; dilutions: 1:500; molecular weight of MYPT-1: 130 kD), rabbit polyclonal p-MYPT-1 antibody (Bioworld, MN, USA; dilutions: 1:500; molecular weight of p-MYPT-1: 130 kD). The membranes were then washed three times in TBS-T and incubated with the corresponding secondary antibody (Santa Cruz, TX, USA; dilutions: 1:10000) conjugated to horseradish peroxidase at room temperature. Immunoreactive bands were visualized with the SuperSignal West Pico enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Band intensities were quantified using a densitometer analysis system (Quantity One; Bio-Rad, Hercules, USA).

Statistics

Values are expressed as mean±SD. Statistical analysis of the results was carried out via one-way analysis of the variance followed by the Newman-Keuls test. Significance was defined at *p*<0.05. The above statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Rat parameters

All rats completed the entire study. The body weight of the sh-

am-group rats did not change significantly from week 0 to week 12, while the weight of the atherosclerosis-group rats was 202±11 g at baseline, although this increased to 242±24 g at week 10. There was no significant difference in body weight between week 10 and week 12.

Changes of lipid profile and CRP

As presented in Table 1, the baseline laboratory variables of the different groups were comparable at the outset, and after 10 weeks of high-cholesterol diet administration, the serum levels of TG, TC, and LDL-C in the atherosclerosis-model groups were significantly greater than those in the sham group (*p*<0.05). Additionally, the serum level of CRP was also profoundly increased in the atherosclerosis-model groups (*p*<0.05 vs. sham group), indicating that atherosclerosis was significantly associated with systemic inflammation. After two weeks of intervention, the serum levels of TC, LDL-C, and CRP in the low-dose darapladib group and the high-dose darapladib group significantly decreased (*p*<0.05 vs. control atherosclerosis group). Darapladib therapy produced no significant changes in HDL-C levels. When compared to the low-dose darapladib group, the reduction of TC, LDL-C, and CRP was more prominent in the high-dose darapladib group (*p*<0.05).

Changes of NO production

As presented in Table 2, after 10 weeks of high-cholesterol diet administration, NO production in the atherosclerosis model groups significantly decreased when compared to the sham group (*p*<0.05). After 2 weeks of darapladib treatment, NO pro-

Table 1. Changes of Lipid Profile and CRP (n=12, each group)

	Control	Atherosclerosis	Low-dose darapladib	High-dose darapladib
Week 0				
TG (mmol/L)	0.93±0.14	0.91±0.17	0.92±0.19	0.92±0.16
TC (mmol/L)	3.01±0.30	3.03±0.26	3.06±0.33	3.02±0.24
LDL-C (mmol/L)	1.99±0.34	2.01±0.35	1.95±0.37	1.99±0.38
HDL-C (mmol/L)	1.01±0.25	0.96±0.26	0.99±0.24	0.99±0.29
CRP (mg/L)	1.25±0.28	1.22±0.29	1.27±0.27	1.24±0.32
Week 10				
TG (mmol/L)	0.95±0.16*	2.11±0.23	2.19±0.29	2.14±0.23
TC (mmol/L)	3.03±0.41*	5.80±0.61	5.92±0.48	5.85±0.76
LDL-C (mmol/L)	1.97±0.48*	3.56±0.50	3.59±0.43	3.59±0.44
HDL-C (mmol/L)	1.01±0.28	0.98±0.29	1.03±0.24	1.01±0.25
CRP (mg/L)	1.24±0.29*	6.96±0.98	6.89±1.03	7.08±0.86
Week 12				
TG (mmol/L)	0.95±0.18*	2.09±0.31	1.92±0.32	1.98±0.30
TC (mmol/L)	3.04±0.44*	5.71±0.57	5.05±0.58 [†]	4.51±0.59 ^{††}
LDL-C (mmol/L)	1.87±0.35*	3.74±0.36	2.92±0.35 [†]	2.53±0.47 ^{††}
HDL-C (mmol/L)	1.03±0.38	1.03±0.27	1.04±0.25	1.05±0.26
CRP (mg/L)	1.25±0.40*	7.14±0.29	4.06±0.68 [†]	3.43±0.67 ^{††}

TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein.

**p*<0.05 vs. other groups, [†]*p*<0.05 vs. atherosclerosis groups, ^{††}*p*<0.05 vs. low-dose darapladib groups.

duction increased when compared to the control group ($p < 0.05$). When compared to the low-dose darapladiB group, the increase of NO production was more prominent in the high-dose darapladiB group ($p < 0.05$).

Changes of Lp-PLA2 activity

As shown in Table 3, after 10 weeks of high-cholesterol diet administration, the serum level of Lp-PLA2 was significantly elevated when compared to the sham group ($p < 0.05$). After 2 weeks of darapladiB treatment, the serum level of Lp-PLA2 was lower than the control atherosclerosis group ($p < 0.05$). When compared to the low-dose darapladiB group, Lp-PLA2 reduction was more prominent in the high-dose darapladiB group ($p < 0.05$).

Effect of darapladiB on cardiomyocyte apoptosis

Fig. 1 showed the TUNEL positive cells in rat hearts of different groups. TUNEL-positive myocytes were expressed as a percentage of total myocytes. The apoptotic rate was $5.16 \pm 0.79\%$ in the control atherosclerosis group. The apoptotic rates were respectively reduced to $4.15 \pm 0.67\%$ and $3.63 \pm 0.59\%$ in the low-dose darapladiB group and the high-dose darapladiB group ($p < 0.05$ vs. control atherosclerosis group). When compared to the low-dose darapladiB group, cardiomyocyte apoptosis reduction was more prominent in the high-dose darapladiB group ($p < 0.05$).

Effect of darapladiB on Rho kinase activity

Rho kinase activity was determined by measuring levels of MYPT-1 phosphorylation, a specific Rho kinase substrate. As shown in Fig. 2, MYPT-1 phosphorylation increased during the control atherosclerosis protocol ($p < 0.05$ vs. sham group). Low-

Table 2. Changes in Serum Level of NO ($\mu\text{mol/L}$) (n=12, each group)

	Control	Atherosclerosis	Low-dose darapladiB	High-dose darapladiB
Week 0	8.63±0.61	8.61±0.84	8.50±0.77	8.60±0.74
Week 10	8.57±0.81*	4.36±0.61	4.50±0.56	4.46±0.66
Week 12	8.79±0.72*	4.55±0.62	6.11±0.56†	6.90±0.64††

NO, nitric oxide.

* $p < 0.05$ vs. other groups, † $p < 0.05$ vs. atherosclerosis groups, †† $p < 0.05$ vs. low-dose darapladiB groups.

Table 3. Changes in Serum Level of Lp-PLA2 (ng/mL) (n=12, each group)

	Control	Atherosclerosis	Low-dose darapladiB	High-dose darapladiB
Week 0	72.99±5.82	75.23±7.69	71.47±8.49	73.58±7.79
Week 10	75.48±6.91*	164.13±13.62	165.23±13.08	166.51±11.94
Week 12	73.16±7.31*	163.97±11.23	145.60±12.59†	129.33±16.32††

Lp-PLA2, lipoprotein-associated phospholipase A2.

* $p < 0.05$ vs. other groups, † $p < 0.05$ vs. atherosclerosis groups, †† $p < 0.05$ vs. low-dose darapladiB groups.

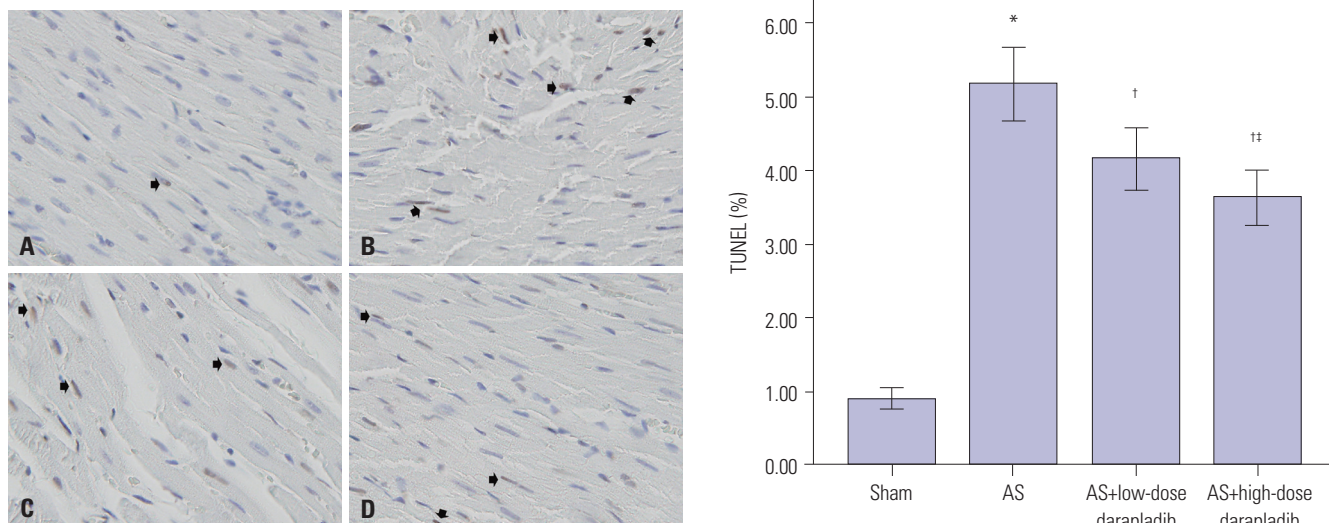


Fig. 1. Representative photomicrographs of ventricular tissue stained via TUNEL for DNA breaks. (A) Sham group. (B) Control atherosclerosis (AS) group. (C) AS+low-dose darapladiB group. (D) AS+high-dose darapladiB group. In the atherosclerosis group an increased number of TUNEL-positive cells was observed (* $p < 0.05$ vs. sham group). After administration of darapladiB, the percentage of TUNEL-positive cells was significantly reduced († $p < 0.05$ vs. control AS group). The percentage of TUNEL-positive cells of the high-dose darapladiB group was significantly lower than the low-dose darapladiB group (†† $p < 0.05$). Black arrows show the TUNEL-positive cells. TUNEL, transferase-mediated dUTP nick end labeling.

dose darapladib therapy resulted in a 23.3% reduction in MYPT-1 phosphorylation ($p < 0.05$ vs. control atherosclerosis group), while high-dose darapladib therapy resulted in a 29.5% reduction ($p < 0.05$ vs. control atherosclerosis group). There was no significant difference between the low-dose darapladib group and the high-dose darapladib group ($p > 0.05$).

DISCUSSION

The results of this study provided experimental evidence that darapladib could reduce elevated Lp-PLA2, Rho kinase activity, and cardiomyocyte apoptosis in atherosclerosis.

Atherosclerosis is a chronic disease with lipids and inflammation playing a crucial role in all stages of the disease from initiation through progression and, ultimately, to thrombotic complications.¹⁸ Atherosclerosis is characterized by increased LDL-C and TC levels, and coronary artery disease is its common manifestation. Previous studies have demonstrated that oxidative stress and apoptosis play a fundamental biological role in atherosclerosis, and it is well documented that NO could diminish oxidative stress and reduce ox-LDL production. In the rats from our experiment, a high-cholesterol diet was found to lead to increased blood lipids, as well as atherosclerotic plaque formation. In the past, numerous biomarkers, such as CRP and cytokines, have been found to be related to atherosclerosis.¹⁹⁻²¹ Previous studies have suggested that Lp-PLA2 is a new biomarker for cardiovascular risk, and Lp-PLA2 activity increases during the development of atherosclerosis.²²⁻²⁵ In our study, we also found that serum Lp-PLA2 activity increased in the atherosclerosis-model group after rats were fed a high-cholesterol diet for 10 weeks, while atherosclerosis significantly reduced levels of NO. Darapladib (a specific inhibitor for Lp-PLA2) reduced TC, LDL-C, and CRP levels and cardiomyocyte apoptosis, as well as Lp-PLA2 activity, in a dose-dependent manner. Additionally, the inhibition of Lp-PLA2 resulted in increase in NO

production. Importantly, NO production was more prominent in high-dose darapladib therapy.

Rho kinase is one of the best-characterized effectors of small GTPase RhoA and plays a crucial role in various cellular functions, such as smooth muscle contraction, cell migration, and cell proliferation. Rho kinase in cardiovascular diseases has received extensive attention and been studied in animal experimental models and human. Rho kinase plays an important role in inflammatory responses and arteriosclerotic lesions in humans and animals;²⁶⁻²⁸ multiple studies have demonstrated that Rho kinase is involved in the inflammatory atherosclerotic process. Rho kinase inhibitors might also result in up-regulation of eNOS and reduce arterial intima-medial thickness, endothelial dysfunction, vascular inflammation, and atherosclerosis plaque formation. This suggests that Rho kinase inhibition may be a potential therapeutic strategy for the prevention of atherosclerosis.

We then attempted to elucidate the relationship between Lp-PLA2 and Rho kinase. We attempted to determine whether the Lp-PLA2 inhibitor could reduce Rho kinase activity in a dose-dependent manner in atherosclerosis. In the present study, the activity of Rho kinase was determined by examining the phosphorylation of MYPT-1. Atherosclerosis resulted in an approximately 46% increase in the amount of p-MYPT-1, indicating the activation of Rho kinase in atherosclerosis. We demonstrated that Rho kinase activity was strongly activated in atherosclerosis. Inhibition of Lp-PLA2 in atherosclerosis led to decreased Rho kinase activity and cardiomyocyte apoptosis. Conversely, a high dose of darapladib ($50 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) did not elicit marked reductions in MYPT-1 phosphorylation when compared to a low dose of darapladib ($25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) in our experimental model. By blocking cholesterol biosynthesis, darapladib also reduced Rho kinase activity in atherosclerosis though not in a dose-dependent manner. There seems to be extensive crosstalk between the NO pathway and Rho kinase. There is convincing evidence that the NO pathway leads to an inhibition of Rho ki-

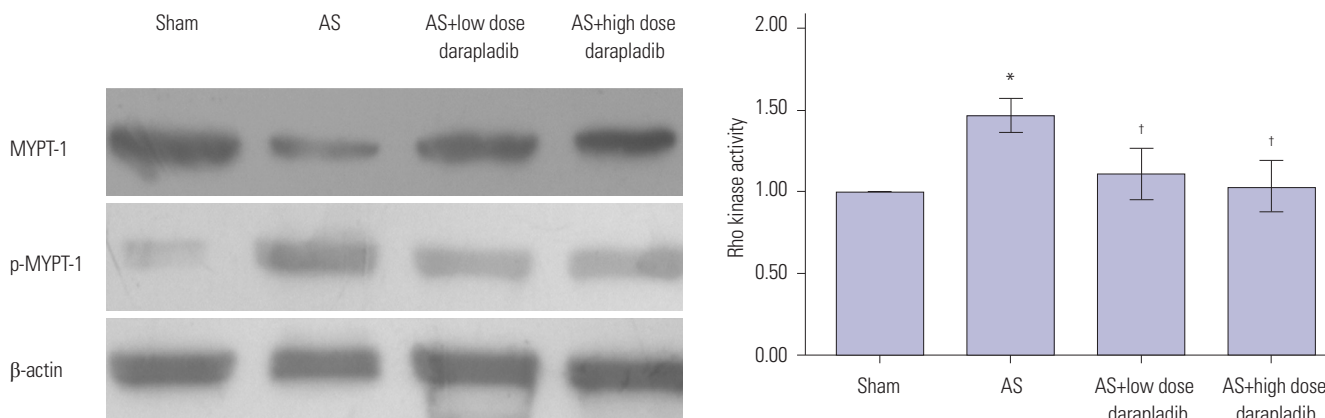


Fig. 2. Western blot analysis of MYPT-1 phosphorylation in rat hearts of different groups (sham, AS, AS+low-dose darapladib, AS+high-dose darapladib). After administration of darapladib, the phosphorylation of p-MYPT-1 was significantly attenuated ($^{\dagger}p < 0.05$ vs. control AS group). There was no significant difference between the low-dose darapladib group and the high-dose darapladib group ($p > 0.05$). $^*p < 0.05$ vs. sham group. MYPT-1, myosin phosphatase targeting subunit 1; AS, atherosclerosis.

nase.²⁹⁻³¹ The elevated Lp-PLA2 level may contribute to impaired NO pathway.³² Thus, we conclude that darapladib may influence Rho kinase activity via the NO pathway.

In summary, the results from our current study show that TG, TC, LDL-C, CRP, Lp-PLA2, Rho kinase activity, and cardiomyocyte apoptosis increased, while NO production was reduced in atherosclerosis *in vivo*. Darapladib, a Lp-PLA2 inhibitor, was found to be beneficial for reducing the CRP level, increasing NO production, and decreasing the Lp-PLA2 level. Darapladib also reduced oxidative stress and cardiomyocyte apoptosis. Furthermore, darapladib not only exerted effects on Lp-PLA2 level but also influenced Rho kinase activity. Darapladib thus leads to cardiovascular protection that might be mediated by its inhibition of both Rho kinase and Lp-PLA2. This may provide a possible new treatment for patients suffering coronary heart diseases in the future.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the research support provided by the Natural Science Foundation of Shandong Province (ZR2011HL069), the Medicine and Science and Technology Planning Project of Shandong Province (2014WS0157), the Natural Science Foundation of Shandong Province (2009ZR019N0), and the Youth Foundation of the Second Hospital of Shandong University (Y2013010010).

REFERENCES

- Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet* 1997;349:1498-504.
- Ocaranza MP, Rivera P, Novoa U, Pinto M, González L, Chiong M, et al. Rho kinase inhibition activates the homologous angiotensin-converting enzyme-angiotensin-(1-9) axis in experimental hypertension. *J Hypertens* 2011;29:706-15.
- Soga J, Noma K, Hata T, Hidaka T, Fujii Y, Idei N, et al. Rho-associated kinase activity, endothelial function, and cardiovascular risk factors. *Arterioscler Thromb Vasc Biol* 2011;31:2353-9.
- Ocaranza MP, Gabrielli L, Mora I, Garcia L, McNab P, Godoy I, et al. Markedly increased Rho-kinase activity in circulating leukocytes in patients with chronic heart failure. *Am Heart J* 2011;161:931-7.
- Zhang J, Bian HJ, Li XX, Liu XB, Sun JP, Li N, et al. ERK-MAPK signaling opposes rho-kinase to reduce cardiomyocyte apoptosis in heart ischemic preconditioning. *Mol Med* 2010;16:307-15.
- Bao W, Hu E, Tao L, Boyce R, Mirabile R, Thudium DT, et al. Inhibition of Rho-kinase protects the heart against ischemia/reperfusion injury. *Cardiovasc Res* 2004;61:548-58.
- Nohria A, Grunert ME, Rikitake Y, Noma K, Prsic A, Ganz P, et al. Rho kinase inhibition improves endothelial function in human subjects with coronary artery disease. *Circ Res* 2006;99:1426-32.
- Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, et al. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J* 1996;15:2208-16.
- Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat Cell Biol* 2001;3:339-45.
- Feng J, Ito M, Kureishi Y, Ichikawa K, Amano M, Isaka N, et al. Rho-associated kinase of chicken gizzard smooth muscle. *J Biol Chem* 1999;274:3744-52.
- Wirth A. Rho kinase and hypertension. *Biochim Biophys Acta* 2010;1802:1276-84.
- Ait-Oufella H, Mallat Z, Tedgui A. [Lp-PLA2 and sPLA2: cardiovascular biomarkers]. *Med Sci (Paris)* 2014;30:526-31.
- Rosenson RS, Hurt-Camejo E. Phospholipase A2 enzymes and the risk of atherosclerosis. *Eur Heart J* 2012;33:2899-909.
- Cai GJ, Miao CY, Xie HH, Lu LH, Su DF. Arterial baroreflex dysfunction promotes atherosclerosis in rats. *Atherosclerosis* 2005;183:41-7.
- Leng X, Zhan R, Wang Y, Liu X, Gong J, Gao X, et al. Anti-heat shock protein 70 autoantibody epitope changes and BD091 promotes atherosclerosis in rats. *Cell Stress Chaperones* 2010;15:947-58.
- Zhang J, Li XX, Bian HJ, Liu XB, Ji XP, Zhang Y. Inhibition of the activity of Rho-kinase reduces cardiomyocyte apoptosis in heart ischemia/reperfusion via suppressing JNK-mediated AIF translocation. *Clin Chim Acta* 2009;401:76-80.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
- Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002;105:1135-43.
- Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med* 2002;347:1557-65.
- Otake H, Shite J, Shinke T, Watanabe S, Tanino Y, Ogasawara D, et al. Relation between plasma adiponectin, high-sensitivity C-reactive protein, and coronary plaque components in patients with acute coronary syndrome. *Am J Cardiol* 2008;101:1-7.
- Ridker PM. C-reactive protein: eighty years from discovery to emergence as a major risk marker for cardiovascular disease. *Clin Chem* 2009;55:209-15.
- Koenig W, Khuseynova N, Löwel H, Trischler G, Meisinger C. Lipoprotein-associated phospholipase A2 adds to risk prediction of incident coronary events by C-reactive protein in apparently healthy middle-aged men from the general population: results from the 14-year follow-up of a large cohort from southern Germany. *Circulation* 2004;110:1903-8.
- Ballantyne CM, Hoogeveen RC, Bang H, Coresh J, Folsom AR, Heiss G, et al. Lipoprotein-associated phospholipase A2, high-sensitivity C-reactive protein, and risk for incident coronary heart disease in middle-aged men and women in the Atherosclerosis Risk in Communities (ARIC) study. *Circulation* 2004;109:837-42.
- May HT, Horne BD, Anderson JL, Wolfert RL, Muhlestein JB, Renlund DG, et al. Lipoprotein-associated phospholipase A2 independently predicts the angiographic diagnosis of coronary artery disease and coronary death. *Am Heart J* 2006;152:997-1003.
- Brilakis ES, McConnell JP, Lennon RJ, Elesber AA, Meyer JG, Berger PB. Association of lipoprotein-associated phospholipase A2 levels with coronary artery disease risk factors, angiographic coronary artery disease, and major adverse events at follow-up. *Eur Heart J* 2005;26:137-44.
- Nunes KP, Rigsby CS, Webb RC. RhoA/Rho-kinase and vascular diseases: what is the link? *Cell Mol Life Sci* 2010;67:3823-36.
- Dong M, Yan BP, Yu CM. Current status of rho-associated kinases (ROCKs) in coronary atherosclerosis and vasospasm. *Cardiovasc Hematol Agents Med Chem* 2009;7:322-30.
- Zhou Q, Gensch C, Liao JK. Rho-associated coiled-coil-forming kinases (ROCKs): potential targets for the treatment of atherosclerosis and vascular disease. *Trends Pharmacol Sci* 2011;32:167-73.
- Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Smolenski A, Lohmann SM, Bertoglio J, et al. Cyclic GMP-dependent protein kinase

- signaling pathway inhibits RhoA-induced Ca²⁺ sensitization of contraction in vascular smooth muscle. *J Biol Chem* 2000;275:21722-9.
30. Chitale K, Webb RC. Nitric oxide induces dilation of rat aorta via inhibition of rho-kinase signaling. *Hypertension* 2002;39(2 Pt 2):438-42.
31. Carter RW, Begaye M, Kanagy NL. Acute and chronic NOS inhibition enhances alpha(2)-adrenoreceptor-stimulated RhoA and Rho kinase in rat aorta. *Am J Physiol Heart Circ Physiol* 2002;283:H1361-9.
32. Tektaş AK, Uslu S, Yalçın AU, Sahin G, Temiz G, Kara M, et al. Effects of lipoprotein-associated phospholipase A2 on arginase/nitric oxide pathway in hemodialysis patients. *Ren Fail* 2012;34:738-43.