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The Recombinant Human Secretory Leukocyte Protease Inhibitor (SLPI) protects cardiac fibroblasts injury against an *in vitro* ischemia/reperfusion injury

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ABSTRACT

One of the major causes of cardiac cell death is an over-secretion of protease enzymes surrounding the ischemic tissue. Therefore, the inhibition of protease activity could be used as an alternative strategy to prevent the expansion of injury. In the present study, we investigated the pre-treatment effects of recombinant human Secretory Leukocyte Protease Inhibitor (rhSLPI) on simulated-ischemia/reperfusion (sI/R)-induced cardiac fibroblast cell death. The adult rat cardiac fibroblasts (ARCFs) were isolated from adult male Wistar rats (n = 6). Isolated cells were characterized by the detection of vimentin. Simulated ischemia/reperfusion was performed by incubating cells in an ischemic buffer for 40 min which was then reperfused with a completed medium for 24 h. The cell viability was determined by MTT assay. The intracellular reactive oxygen species (ROS) production was measured by dichlorodihydrofluorescein assay. Activation of p38 MAPK and apoptotic proteins was determined by Western blotting. The results showed that pre-treatment of rhSLPI at the concentrations of 400, 600, 800, and 1000 ng/mL significantly reduced sI/R-induced ARCFs death as well as intracellular ROS production. Moreover, pre-treatment of rhSLPI could reduce p38 MAPK activation and pro-apoptotic protein-Bax levels. In conclusion, pre-treatment of rhSLPI shows cardioprotective effects against sI/R-induced cardiac fibroblast cell death by reducing oxidative stress and reducing p38 MAPK activation.

INTRODUCTION

One of the key mechanisms of cardiac cell death during myocardial ischemia/reperfusion (I/R) injury is the overproduction of protease enzymes, which can be secreted from not only infiltrated leukocytes but also cardiomyocytes and cardiac fibroblast itself (Boudoulas and Hatzopoulos, 2009; Epelman *et al*., 2015). These protease enzymes cause cellular necrosis, both located within and around the ischemic area (Jordan *et al*., 1999). Therefore, prevention or attenuation of the protease enzyme activity caused by myocardial I/R injury is one of the most promising therapeutic targets for ischemic heart disease (IHD). The beneficial effects of rhSLPI in ischemia/reperfusion injury was reported in cardiac transplantation (Schneeberger *et al*., 2008). In addition, our unpublished data in an *in vitro* model of cardiomyocytes showed that exogenous treatment of recombinant human Secretory Leukocyte Protease Inhibitor (rhSLPI) could protect cardiac cells from sI/R injury. A similar outcome was also exhibited in vascular endothelial cell injury from ischemia/reperfusion insults (Prompunt, 2016; Nernpermpisooth *et al*., 2017). In fact, the heart consists of approximately 70% non-myocytes and 30% cardiac myocytes (Brilla *et al*., 1993). The cardiac fibroblast is in the non-cardiomyocyte population in the heart. Cardiac fibroblast not only plays role in cardiac development and remodeling, but it also regulates cardiomyocytes function and viability via cell-cell interaction (Torre-Amione *et al*., 1996). It has been known that I/R injury causes cardiac cell death and could induce cardiac fibroblast injury and finally result in myocardial fibrosis (Chen and Frangogiannis, 2013). Thus, any strategy that is capable of

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protecting cardiac fibroblast from I/R injury could also protect cardiomyocytes. However, the cytoprotective effects of rhSLPI have never been investigated in sI/R-induced cardiac fibroblast cell death. Therefore, the aim of this study is to investigate the *in vitro* cytoprotective effects of rhSLPI on cardiac fibroblast subjected to simulated ischemia/reperfusion (sI/R) injury.

MATERIALS AND METHODS

Adult rat cardiac fibroblast isolation and culture

Adult Rat Cardiac Fibroblasts (ARCFs) were isolated from adult male Wistar rats (200-250 g) (n = 6) by collagenase-based enzymatic digestion using an adaptation of the method used by Kumphune *et al*. (Kumphune *et al*., 2010). The study protocol was approved by Naresuan University Animal Ethics committee (protocol license No.55 04 0005). The rats were anesthetized by intraperitoneal injection (IP) with pentobarbital (300 mg/kg) and heparin (150 units). The rat hearts were excised and initially perfused for 5 min with modified Krebs solution (solution A) containing 130 mM NaCl, 4.5 mM KCl, 1.4 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 0.75 mM $CaCl_2$, 4.2 mM HEPES, 20 mM taurine, 10 mM creatine and 10 mM glucose, pH 7.3 at 37°C). The hearts were then perfused with a calcium-free solution containing 100 µM EGTA for 5 min, followed by perfusion with solution A containing 100 µM $CaCl_2$ and 0.4 mg/ml Worthington type II collagenase for 10 min. The ventricles were then cut into small pieces, which were incubated in 10 ml of collagenase solution gassed with 100% O_2 for a further 7 min at 37°C, with regular triturating. Then isolated myocytes were allowed to settle into a loose pellet and the supernatant containing cardiac fibroblasts were collected for further study. The ARCFs were resuspended with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 unit/mL of penicillin and 100 µg/mL streptomycin and allowed to settle for at least 2 h before removal of non-adherence cells. The adhered cardiac fibroblasts were cultured in complete medium for further experiments. Isolated cells were determined for fibroblast characteristics by used an immunofluorescence technique with a specific antibody against Vimentin (1:1000, Abcam).

Simulated ischemia (sI) and determination of cell viability

Simulated ischemia (sI) was induced by incubating ARCFs with a specifically modified Krebs-Henseleit buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, and 4.0 mM HEPES) with 20 mM 2-deoxyglucose, 20 mM sodium lactate, and 1 mM sodium dithionite at pH 6.5. The control buffer was composed of Krebs-Henseleit buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, and 4.0 mM HEPES), supplemented with 20 mM D-glucose, 1 mM sodium pyruvate. Simulated ischemia (sI) was performed for 40 min. After simulated ischemia was achieved, the ischemic buffer or control buffer was removed and the cells were subjected to reperfusion (sI/R) by replacing the ischemic buffer with complete medium and then incubated at 37°C, 5% CO_2 for 24 h.

Determination of cell viability

ARCFs were cultured and treated with various concentrations of rhSLPI (Sino Biological, Inc., Beijing, China) at concentration of 0 ng/ml, 200 ng/ml, 400 ng/ml, 600 ng/ml, 800 ng/ml, and 1,000 ng/ml, for 24 h before being subjected to 40 min of sI/R. At the end of reperfusion, the culture media was removed. Then, 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) of reagent was added and incubated for 2 h at 37°C. The formazan dye was finally solubilized with 500 µl of Dimethyl sulfoxide (DMSO). The formazan dye was collected and the optical density (OD) was determined by a spectrophotometer at λ 490 nm using DMSO as a blank. The relative percentage of cell viability was compared to the control group.

In addition, cell viability was also determined by using a trypan blue dye exclusion method. After the sI/R protocol, cells were then gently harvested and mixed with 0.4% trypan blue solution (Sigma, USA). The resulting cell suspension was counted by Countess II FL Automated Cell Counter (Invitrogen). The viable cells were expressed as a percentage of the total counted cells.

Measurements of cellular injury

The released-Lactate dehydrogenase (LDH) activity assay kit used in this study is a modified method based on the recommendations of the Scandinavian Committee on Enzymes (LDH SCE mod.). The kit was purchased from HUMAN (Wiesbaden, Germany). Ten microliters of culture medium were mixed with 1000 µl of reaction buffer and incubated at 37°C for 5 min. Then, 250 µl of substrate reagent was added. The solution was mixed and the absorbance read after 1 min at ʎ 340 nm. The mean absorbance change per minute (ΔA/min) was used to calculate LDH activity by the following formula;

$$\text{LDH activity (U/l)} = \Delta A/\text{min} \times 20{,}000$$

Determination of cellular reactive oxygen species (ROS)

ARCFs at a concentration of 1×10^5 cells/mL were cultured in a 96-well black plate at 37°C, 5% CO_2, and 95% O_2 until it reached 80% confluence. The culture media were removed and the cells were washed once with PBS prior to incubating with complete media containing 250 µM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) in a dark room for 30 min at 37°C. The medium containing carboxy-H2DCFDA was then removed and the cells were washed with PBS. For rhSLPI treatment, 200 µl of completed medium containing various concentrations of rhSLPI were added and incubated for 1 h at 37°C. Then, 250 µM hydrogen peroxide (H_2O_2) was applied to the cells and incubated for 30 min at 37°C. The ROS activity was determined by measuring the fluorescence intensity with an EnSpire Multimode Plate Reader (PerkinElmer, Massachusetts, USA). The filter suitable for detecting the signal gave the excitation wavelength at λ 498 nm and emission wavelength at λ 522 nm. The result was expressed in Arbitrary Unit (A.U.).

Measurement of p38 MAPK activation and apoptosis regulatory proteins by Western blot analysis

ARCFs cells were extracted in cold Radioimmunoprecipitation assay buffer (RIPA buffer). After centrifugation at 14,000 g for 15 min at 4°C, the supernatants were collected, and then separated on 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to

polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skimmed milk, the membranes were blotted with antibodies against total p38 MAPK, phosphorylated-p38 MAPK, bcl-2-like protein 4 (Bax), B-cell lymphoma 2 (Bcl-2), β-actin (all from Cell Signaling, USA) using the Luminata™ Western HRP Substrates system (Merck, USA). The images were captured and quantified with Image Lab 4.0 software (Bio-Rad Laboratories), and the values were normalized to β-actin.

Statistical analysis

All values were expressed as mean ± standard error of the mean (S.E.M). All comparisons were assessed for significance using One-way Analysis of variance (ANOVA), followed when appropriate by the Tukey-Kramer test. The statistical test was performed using commercially available software (Lab chart Prism version 5). A p-value of less than 0.05 was considered as statistically significant.

RESULTS

Characterization of isolated ARCFs

The results showed that isolated ARCFs showed the morphology of fibroblast cells with a flat, spindle shape with multiple processes spreading from the main body and lack of basement membrane (Figure 1A). Immunostaining of Vimentin, cell surface molecule specifically for fibroblast, showed that the isolated ARCFs exhibited Vimentin staining throughout the cell surface and this did not appear in the nucleus (Figure 1B). In addition, the determination of Vimentin expression by Western blot analysis showed that isolated ARCFs could express Vimentin protein at the molecular weight of 54 kDa (Figure 1C). These results suggested that the isolated cells from ventricle expressed fibroblast phenotypes.

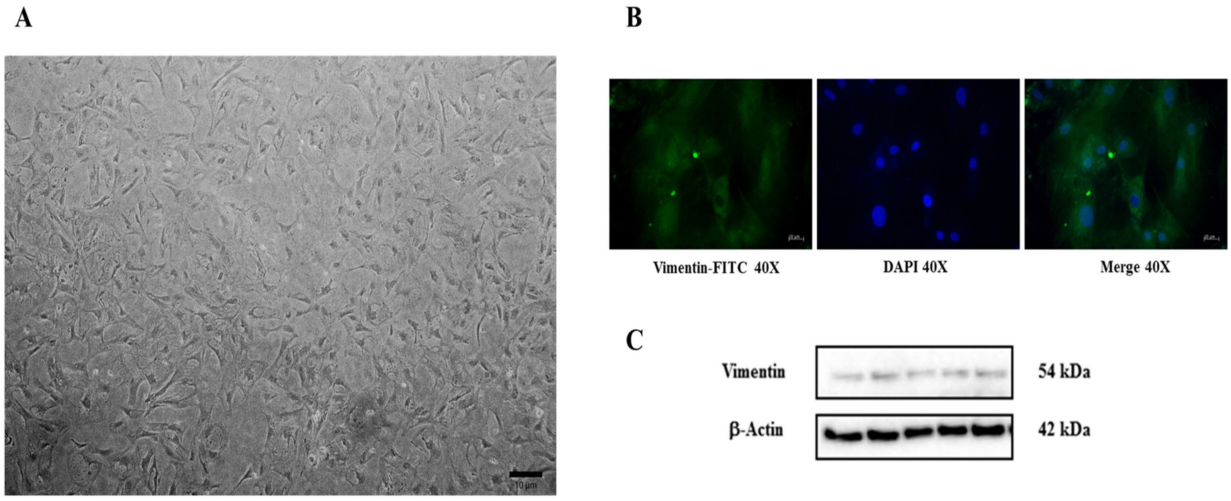

Fig. 1: Characterization of ARCFs morphology and expression of marker protein (Vimentin). (A) showed cell morphology observed under the bright field inverted-microscope. (B) ARCFs were stained with Fluorescein isothiocyanate (FITC)-conjugated Vimentin (green) and the nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) (blue). (C) Western blot of adult rat cardiac fibroblasts showing expression of biological marker (Vimentin) at 54 kDa, each lane represented ARCFs independently isolation.

Treatment of rhSLPI in ARCFs reduced cell death and ROS production during I/R injury

The results showed that sI/R significantly decreased cell viability (53.89 ± 4.08%). Pre-treatment of rhSLPI prior to sI/R at concentrations of 400 ng/mL (64.44 ± 2.08%), 600 ng/mL (67.50 ± 4.88%), 800 ng/mL (68.06 ± 5.41%) and 1000 ng/mL (69.31 ± 4.92%), significantly increased cell viability of the ARCFs when compared to the sI/R group (53.89 ± 4.08%) ($p < 0.05$) (Figure 2A). Similar findings could also be observed in trypan blue dye exclusion assay. The results showed that pre-treatment of rhSLPI at concentrations greater than 200 ng/ml significantly increased cell viability (Figure 2B) and reduced cellular injury by released-LDH activity (Figure 2D). Treatment of rhSLPI in all concentrations could not reduce cell viability or increase cellular injury (Figure 2C, E).

rhSLPI reduced I/R-induced intracellular ROS production

To further investigate the cardioprotective effects of rhSLPI treatment during I/R injury, we next determined the effect of rhSLPI on intracellular ROS generation. The results showed that there was an increase in intracellular ROS production in the ARCFs after challenging with H_2O_2 when compared to the control (28470 ± 3827 A.U. versus 2280 ± 308.2 A.U.). Pre-treatment of rhSLPI at concentrations of 600 ng/mL (21500 ± 2002 A.U.), 800 ng/mL (21620 ± 2348 A.U.) 1,000 ng/mL (21070 ± 2571 A.U.) could significantly reduce intracellular ROS production ($p < 0.05$). However, pre-treatment of rhSLPI at 200 ng/mL and 400 ng/mL did create a slight decrease in intracellular ROS production (Figure 3).

Treatment of rhSLPI in ARCFs attenuated p38 MAPK phosphorylation and apoptotic regulatory protein

Myocardial ischemia/reperfusion injury is a potent stimulant of p38 MAPK activation, which leads to myocardial cell death by apoptosis and myocardial cell injury (Kumphune *et al*., 2010). To determine the activation of p38 MAPK, Western blot analysis was performed in rhSLPI treatment prior to sI. The Western blot analysis was performed by using the specific antibody against phosphorylated p38 MAPK.

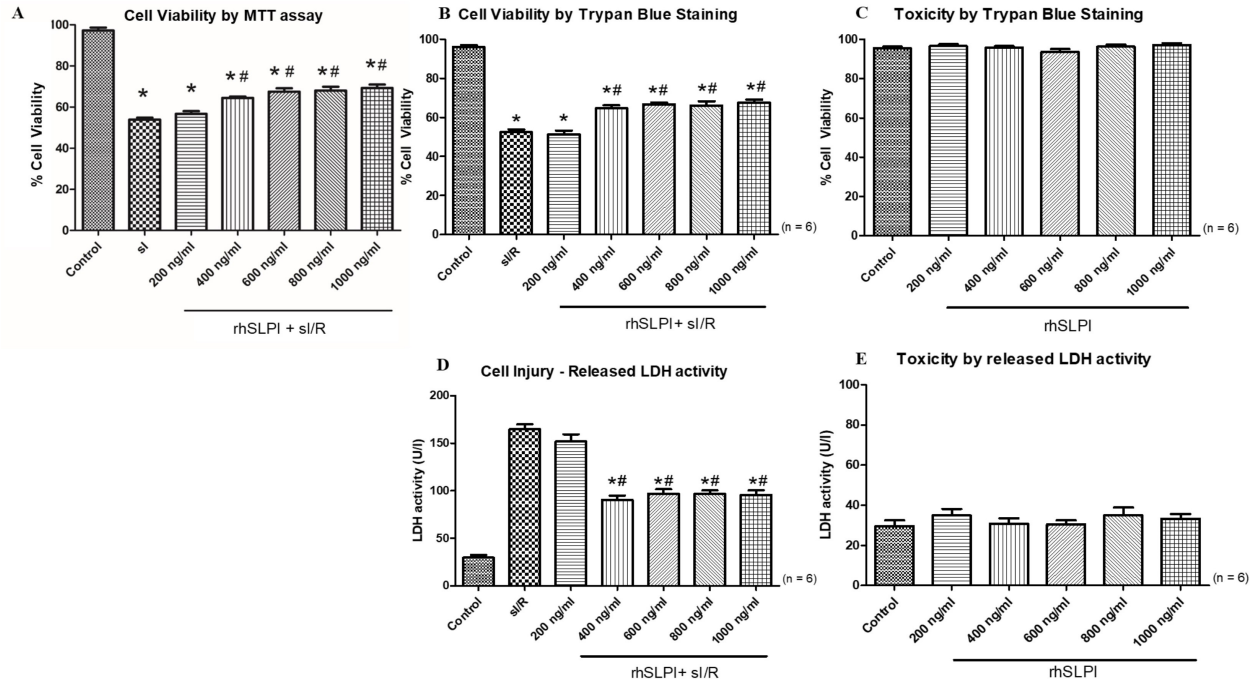

Fig. 2: Determination the effect of the rhSLPI treatment in ARCFs subjected to sI/R injury. (A) The percentage of cell viability by MTT assay of pre-treatment of ARCFs with various concentrations of rhSLPI. (B, C) Cell viability by trypan blue dye exclusion assay. (D, E) Cellular injury by released-LDH activity assay. Each bar graph represents means ± S.E.M for each the 3 experiments. *p < 0.05 versus control group (ANOVA), #p < 0.05 versus sI groups (ANOVA).

The result showed that p38 MAPK was strongly activated in response to simulated ischemia. Pre-treatment with rhSLPI at 1,000 ng/mL significantly reduced p38 MAPK phosphorylation when compared to sI group (Figure 4A, B). In addition, the apoptotic regulatory proteins Bcl2 and Bax were also determined. The results showed that pre-treatment with SLPI could significantly reduce Bax expression (Figure 4A, D), but not Bcl2 (Figure 4A, C). These results indicated that the treatment of rhSLPI in ARCFs could potentially attenuate p38 MAPK activation and Proapoptotic protein Bax expression.

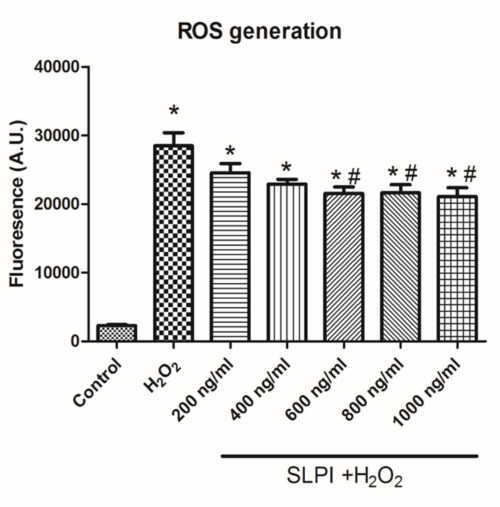

Fig. 3: The figure shows reducing intracellular ROS production. Each bar graph represents means ± S.E.M for each the 3 experiments. *p < 0.05 versus control (ANOVA), #p < 0.05 versus H_2O_2 treated group (ANOVA).

DISCUSSION

The present study highlights the cytoprotective effects of recombinant human secretory leukocyte protease inhibitor (rhSLPI) on an *in vitro* simulated ischemia/reperfusion (sI/R)-induced cardiac fibroblast injury. The major findings of this study were the pre-treatment of rhSLPI prior to an *in vitro* sI/R could reduce sI/R-induced cardiac fibroblast cell death, intracellular ROS production, ischemic-induced p38 MAPK phosphorylation, and pro-apoptotic protein Bax.

The cardiac fibroblast plays a role as a sentinel cell by constructing scaffolding to hold other cells together (Nag, 1980). Fibroblasts play a key role after infarction as the outcome depends on the generation of a fibrous scar comprised largely of collagen (Sun and Weber, 2000). In the previous study, the secretome of cardiac fibroblast could reduce cardiomyocytes death in co-culture models, suggesting that there is communication between cardiomyocytes and cardiac fibroblast (Abrial *et al*., 2014). During I/R injury, it has been known that various protease enzymes were actively secreted from injured cardiac resident cells. These enzymes could harm not only the cells that secrete the protease itself but also injure the surrounding cardiomyocytes or cardiac fibroblast cells. These could expand the ischemic area as well as the infarct size (Jordan *et al*., 1999; Boudoulas and Hatzopoulos, 2009; Epelman *et al*., 2015). In addition, leukocytes and neutrophils infiltrate the ischemic area during reperfusion and secrete various serine protease enzymes, including cathepsin G, elastase, and trypsin (Kuckleburg and Newman, 2013). Necrotic cells also secrete intracellular protease enzymes, which contribute to heart tissue damage and ultimately impair cardiac function (Jordan *et al*., 1999; Boudoulas and Hatzopoulos, 2009; Epelman

et al., 2015). Therefore, inhibiting protease activity may be a key strategy for preventing cardiac tissue injury following myocardial ischemia. We previously reported that rhSLPI overexpression in cardiomyocyte or rhSLPI treatment in isolated hearts could significantly reduce cell death, injury, as well as reduced infarct size (Prompunt, 2016). In addition, rhSLPI treatment could reduce ischemia-induced vascular endothelial cell death and injury (Nernpermpisooth *et al*., 2017). However, the protective effect of rhSLPI on cardiac fibroblast sI/R injury has never been investigated. We hypothesized that rhSLPI could protect cardiac fibroblast from sI/R injury *in vitro*, which could possibly protect the heart from ischemic injury as well as limit post-infarction remodeling.

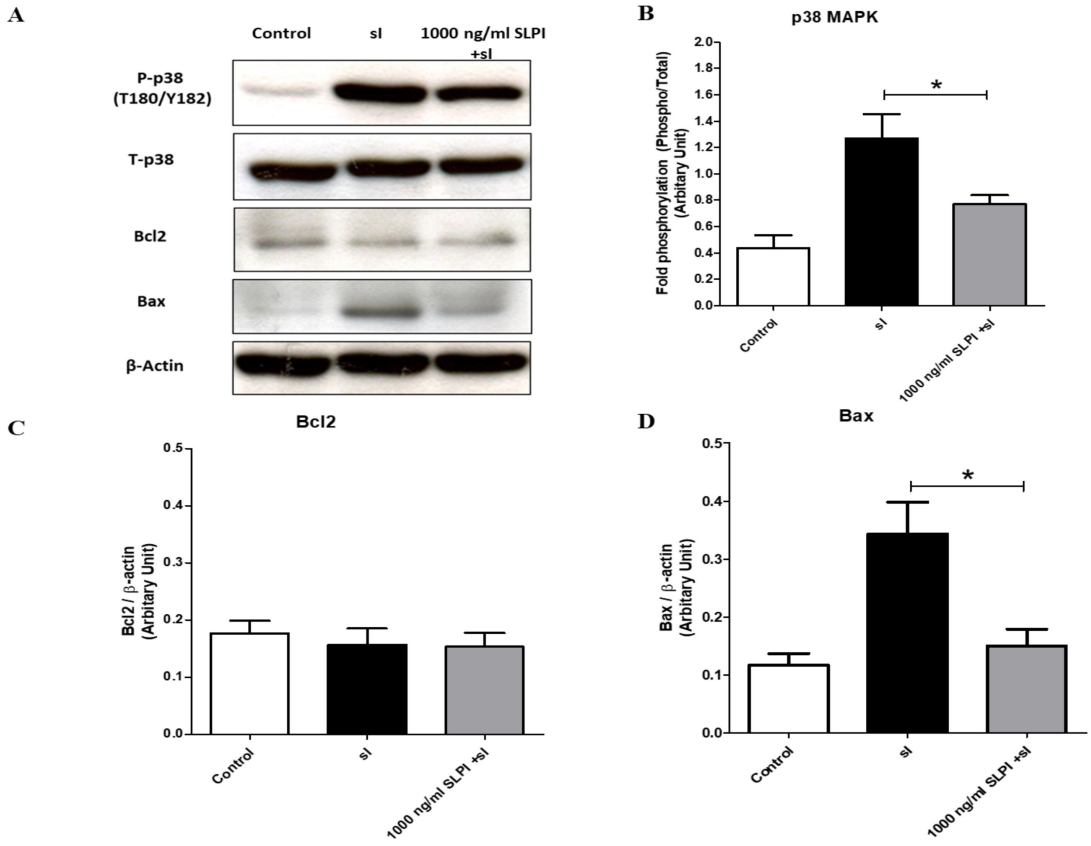

Fig. 4: Determination of cellular signaling in response to treatment of ARCFs with rhSLPI, subjected to sI/R. ARCFs were treated with rhSLPI at 1,000 ng/mL for 2 h before subjected to sI. The activation of p38 MAPK (A, B), anti-apoptotic protein Bcl2 (A, C), and proapoptotic protein Bax (A, D), was observed by Western blot analysis.

Recently, a new role of cardiac fibroblasts was discovered, in that, they act as sentinel cells that sense danger signals and enhance the inflammatory response to I/R (Kawaguchi *et al*., 2011). Under hypoxia/reoxygenation, inflammatory response was in cardiac fibroblasts through ROS production. In the present study, rhSLPI exhibited an antioxidant property to attenuate H_2O_2-induced cardiac fibroblast intracellular ROS production in a dose-dependent manner. In addition, several reports demonstrated that SLPI treatment in several cell types could reduce inflammation and also showed anti-apoptotic effects (Seto *et al*., 2009; Subramaniyam *et al*., 2011). Reactive oxygen species (ROS) are responsible for the myocardial damage during both ischemia and the reperfusion period. The generation of ROS can be an indicator of apoptosis (Braunersreuther and Jaquet, 2012; Zhou *et al*., 2015). The reduction of intracellular ROS by rhSLPI treatment could be a crucial mechanism to regulate apoptosis in this I/R injury. One of the possible explanations of anti-apoptotic effect of rhSLPI through modulation of intracellular ROS production. The excessive formation of ROS during I/R injury induces cardiac cell death directly by inducing cell membrane and protein damage (Hoffman *et al*., 2004) or indirectly by activating pro-apoptotic pathways (Ferrari, 1998; Hoffman *et al*., 2004). Proapoptotic Bax activation plays an important role in the apoptotic process. In this study, we found that rhSLPI treatment could significantly reduce Bax expression, although the anti-apoptotic protein, Bcl-2, was not altered. Therefore, rhSLPI that mediated more intracellular ROS reduction would attenuate more cardiac fibroblast apoptosis and subsequently limit further injury, which is crucial for infarcted area expansion, myocardial fibrosis and dysfunction (Kawaguchi *et al*., 2011).

The p38 MAPK is activated during cellular stresses, including I/R injury. Ischemia is a potent stimulant of p38 MAPK activation (Kumphune *et al*., 2012). Inhibition of p38 MAPK during ischemia reduces infarction and inhibits the production of inflammatory cytokines, such as Tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-8 (IL-8), which are known to aggravate ischemic injury (See *et al*., 2004; Clark *et al*., 2007; Kumphune *et al*., 2010; Kumphune *et al*., 2012). Moreover,

reperfusion can re-activate p38 MAPK, perhaps in response to ROS stimulation (Son *et al*., 2013). Interestingly in sI/R, pre-treatment cardiac fibroblast with rhSLPI reduced cell death; this was hypothesized that the decrease in cardiac fibroblast cell death following the treatment with rhSLPI was due to attenuation of p38 MAPK activation. The results of the current study indicated that the administration of rhSLPI prior to sI significantly attenuated the phosphorylation of p38 MAPK, and therefore protected cardiac fibroblast cells against sI/R injury.

There are several points that could be considered as limitations of this study. One of the interesting issues to consider is the fact that the results from this study were obtained solely from an *in vitro* study on the cytoprotective effects of rhSLPI in cardiac fibroblast cells subjected to sI/R. Further investigation should be performed to overexpress the rhSLPI gene in cardiac fibroblast, which then co-culture with cardiomyocytes in an attempt to determine if the secretion of rhSLPI from cardiac fibroblast could potentially protect cardiomyocytes. This could provide more information and is closely related to the real physiology of the heart.

CONCLUSIONS

In conclusion, we firstly demonstrated the cytoprotective effects of rhSLPI on primary isolated adult rat cardiac fibroblasts subjected to sI/R. The cytoprotective effects of rhSLPI could be due to the reduction of intracellular ROS production from sI/R injury. In addition, pretreatment of rhSLPI could reduce cell death by attenuation of p38 MAPK phosphorylation and reduction of pro-apoptotic protein Bax expression.

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AUTHOR CONTRIBUTIONS

Nitchawat Paiyabhroma and Sarawut Kumphune conceived and designed the experiments; Nitchawat Paiyabhroma performed the experiments; Nitchawat Paiyabhroma and Sarawut Kumphune analyzed the data; Sarawut Kumphune contributed reagents/materials/analysis tools; Nitchawat Paiyabhroma, Nitirut Nernpermpisooth, and Sarawut Kumphune wrote and prepared the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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