The cardioprotective effects of secretory leukocyte protease inhibitor against myocardial ischemia/reperfusion injury

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Abstract. Protease enzymes generated from injured cells and leukocytes are the primary cause of myocardial cell damage following ischemia/reperfusion (I/R). The inhibition of protease enzyme activity via the administration of particular drugs may reduce injury and potentially save patients' lives. The aim of the current study was to investigate the cardioprotective effects of treatment with recombinant human secretory leukocyte protease inhibitor (rhSLPI) on *in vitro* and *ex vivo* models of myocardial I/R injury. rhSLPI was applied to isolated adult rat ventricular myocytes (ARVMs) subjected to simulated I/R and to *ex vivo* murine hearts prior to I/R injury. Cellular injury, cell viability, reactive oxygen species (ROS) levels, and levels of associated proteins were assessed. The results demonstrated that administration of rhSLPI prior to or during sI/R significantly reduced the death and injury of ARVMs and significantly reduced intracellular ROS levels in ARVMs during H_2O_2 stimulation. In addition, treatment of ARVMs with rhSLPI significantly attenuated p38 mitogen-activated protein kinase (MAPK) activation and increased the activation of Akt. Furthermore, pretreatment of *ex vivo* murine hearts with rhSLPI prior to I/R significantly decreased infarct size, attenuated p38 MAPK activation and increased Akt phosphorylation. The results of the current study demonstrated that treatment with rhSLPI induced a cardioprotective effect and reduced ARVM injury and death, intracellular ROS levels and infarct size. rhSLPI also attenuated p38 MAPK phosphorylation and activated Akt phosphorylation. These results suggest that rhSLPI may be developed as a novel therapeutic strategy of treating ischemic heart disease.

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Introduction

Despite advances in knowledge and technology regarding health, myocardial ischemia remains the most common cause of mortality worldwide and the number of people succumbing to myocardial ischemia is predicted to increase over the coming decades (1,2). In the majority of cases, myocardial infarction is a consequence of coronary occlusion that results in an insufficient blood supply to the myocardium, leading to irreversible necrosis (3). Restoration of blood flow of the ischemic region, known as reperfusion, is the most effective therapeutic strategy of rescuing myocardial cells and saving patients' lives. However, as it results in the abrupt restoration of the oxygen supply, myocardial reperfusion itself may aggravate myocardial injury, leading to reperfusion injury (4). During myocardial ischemia and reperfusion, cardiac cell death, calcium overload and mitochondrial permeability transition pore (mPTP) opening occur, resulting in the generation of reactive oxygen species (RO) (5-7). Various protease enzymes are secreted during ischemia/reperfusion (I/R) injury. These enzymes are generated from neighboring cells that are damaged following I/R injury, leading to increased cell death and the injury of normal cardiomyocytes surrounding the ischemic area (5-7). In addition, leukocytes and neutrophils infiltrate the ischemic area during reperfusion and secrete serine various protease enzymes, including cathepsin G, elastase and trypsin (8). Necrotic cells also secrete intracellular protease enzymes,

which contribute to heart tissue damage and ultimately impair cardiac function (5-7). Therefore, inhibiting protease activity may be a method of preventing cardiac tissue injury following myocardial ischemia.

Secretory leukocyte protease inhibitor (SLPI) is an 11.7 kDa long cationic non-glycosylated protein that belongs to the Whey Acidic Protein (WAP) family (9-11). SLPI inhibits numerous leukocyte serine proteases, including trypsin and chymotrypsin in pancreatic acinar cells, elastase and cathepsin G in neutrophils, and chymase in mast cells (10-12). SLPI is a frontline protein that directly and indirectly defends against infection by viruses, bacteria and fungi (10). SLPI also acts as anti-inflammatory mediator, protecting host tissue from the excessive tissue damage induced by proteolytic enzymes secreted during inflammation (10). Therefore, SLPI may be a novel therapeutic strategy to treat myocardial ischemia.

Schneeberger *et al (*13) reported on the effects of rhSLPI in cardiac transplantation and demonstrated that supplementation of recombinant human (rh)SLPI in the cold-preservative solution used to preserve hearts prior to transplant improves the cardiac score of these hearts following transplantation. rhSLPI serves a crucial role in early myocardial performance and post-ischemic inflammation following cardiac transplantation (13). However, the immediate effects of rhSLPI on myocardial I/R injury at the cellular and molecular levels have not yet been investigated. Therefore, rhSLPI may be a developed as a cardioprotective agent to treat myocardial I/R.

Therefore, the aim of the current study was to investigate the effect of rhSLPI on isolated adult rat ventricular myocytes (ARVMs) subjected to simulated I/R and to investigate the cardioprotective effects of rhSLPI on an *ex vivo* ischemia/reperfusion isolated murine heart model.

Materials and methods

Reagents. All basic chemicals were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). M199 medium was obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). For SDS-PAGE and western blot analysis, 30% polyacrylamide solution was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) and the polyvinylidenedifluoride (PVDF) membrane was purchased from GE Healthcare Life Sciences (Little Chalfont, UK). The antibodies for the dual phosphorylated (p)-Thr180/Tyr182 form of p38 mitogen-activated protein kinase (MAPK; cat. no. sc-17852-R), total p38 MAPK (cat. no. sc-728), p-Akt (cat. no. sc-293125) and total Akt (cat. no. sc-8312) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Enhanced chemiluminescence (ECL) solution was purchased from GE Healthcare Life Sciences and collagenase type II was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). rhSLPI was purchased from Sino Biological, Inc. (Beijing, China).

Experimental animals. Adult male Wistar rats (8 weeks old) weighing 200-250 g (n=6) and adult male C57BL/6 mice (8 weeks old) weighing 25-30 g (n=18) were obtained from the National Animal Center, Salaya Campus, Mahidol University (Bangkok, Thailand). All animals were maintained under environmentally controlled conditions (a temperature of 22±1˚C, humidity of 45-60% and 12-h light/dark cycle) at the Center for Animal Research, Naresuan University (Phitsanulok, Thailand). All protocols used in the current study were approved by the animal ethics committee of the Center for Animal Research, Naresuan University (approval no. NU-AE550732).

Isolation of ARVMs and culture. The Wistar rats were euthanized by intraperitoneal injection (IP) with pentobarbital sodium (Nembutal® Sodium Solution CII; 100 mg/kg; Akorn, Inc., Lake Forest, IL, USA) and lithium heparin (150 U; Government Pharmaceutical Organization, Bangkok, Thailand). Adult rat ventricular myocytes (ARVMs) were isolated from the hearts using collagenase-based enzymatic digestion. Hearts were excised and initially perfused for 10 min with a 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 at pH 7.3 at 37˚C. Hearts were then perfused with a calcium-free solution containing 100 μM EGTA for 5 min (solution B), followed by perfusion with solution A containing 100 μM $CaCl_2$ and 0.4 mg/ml Type II collagenase for 30 min at 37˚C. Following enzymatic perfusion, ventricles were cut into small pieces, which were incubated in 20 ml collagenase solution. Ventricles were incubated with 100% O_2 for a further 7 min at 37˚C and underwent regular gentle triturating. Isolated cardiomyocytes were separated from undigested ventricular tissue using a cell strainer. Subsequently, isolated myocytes were allowed to pellet at the bottom of the tube (sedimentation by gravity) and the supernatant was removed and replaced with solution A containing 1% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) and 500 μM $CaCl_2$. Isolated cardiomyocytes were then allowed again to sediment at the bottom of the tube (sedimentation by gravity) and the supernatant was then removed and replaced with 10 ml solution A containing 1 mM $CaCl_2$. The cell pellet was washed with M199 culture medium containing 100 IU/ml penicillin/streptomycin. Myocytes were resuspended in M199 supplemented with 2 mM creatine, 2 mM carnitine and 5 mM taurine, and then seeded on pre-laminin-coated 6-well plates (15 μg/ml laminin). Myocytes were allowed to adhere for 2 h in an incubator containing 5% CO_2 at 37˚C. The culture medium was replenished with fresh modified M199 medium, prior to further experiments.

Simulated ischemia (sI) protocol. sI was performed following a modified protocol (14). ARVMs were incubated with a specific basic buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$ and 4.0 mM HEPES), 20 mM 2-deoxyglucose, 20 mM sodium lactate and 1 mM sodium dithionite at pH 6.5, in order to induce sI. The control buffer was composed of the basic 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 and 1 mM sodium pyruvate. ARVMs subjected to 20 min sI and 2 h reperfusion (sI/R) were treated with 0, 1, 10, 100, 1,000 and 10,000 ng/ml rhSLPI. The treatment was applied either 2 h prior to sI, during sI or at the onset of reperfusion. Following reperfusion, the culture medium was collected to assess lactate dehydrogenase (LDH) activity and cell viability was determined using the trypan blue dye

exclusion assay. Another set of experiments was performed to determine the most effective concentration of rhSLPI. Cells were treated with 0, 200, 400, 600, 800 and 1,000 ng/ml rhSLPI prior to or during sI. Subsequently, cell viability was determined using the trypan blue dye exclusion assay and cell injury was assessed by determining released-LDH activity.

Determination of cell viability. The viability of ARVMs was assessed using the trypan blue dye exclusion method. Following reperfusion, the culture medium was removed and cells were incubated in 0.4% trypan blue solution for 1-2 min at room temperature. Following incubation, the numbers of trypan blue-positive and negative cells were counted under a light microscope in 5 different microscopic fields. The percentage of surviving cells from the total amount of cells was then calculated and the relative percentage of cell viability was compared with the control group.

Measurement of cellular injury. Released-Lactate Dehydrogenase (LDH) enzyme activity was measured by the LDH SCE mod. liquiUV kit (cat. no. 12214; HUMAN Diagnostics Worldwide, Wiesbaden, Germany). A total of 10 μl culture medium was mixed with 1,000 μl LDH activity assay buffer and incubated at 37˚C for 5 min. Then, 250 μl substrate reagent was added. The solution was mixed and after 1 min, absorbance was measured at a wavelength of 340 nm. The mean absorbance change per minute (ΔA/min) was measured to evaluate released-LDH activity using the following formula: LDH activity (U/I)=ΔA/min x 20,000.

Measurement of intracellular ROS level production. ARVMs were cultured with modified M199 medium in 24-well black plates and maintained at 37˚C in an atmosphere containing 5% CO_2 and 95% O_2. The culture medium was removed and the cells were washed with phosphate-buffered saline (PBS). Subsequently, cells were incubated with M199 medium containing 25 μM carboxy-H2DCFDA (Sigma-Aldrich; Merck KGaA) in a dark room for 30 min at 37˚C. The medium containing carboxy-H2DCFDA was discarded and ARVMs were washed once with PBS. For rhSLPI treatment, 500 μl M199 medium containing various concentrations of rhSLPI (0, 200, 400, 600, 800 and 1,000 ng/ml) was added and incubated for 1 h at 37˚C. Subsequently, cells were incubated with 250 μM H_2O_2 for 30 min at 37˚C. The control cells were incubated with M199 medium without treatment. ROS activity was determined by measuring fluorescence intensity using an EnSpire Multimode Plate Reader (PerkinElmer, Inc., Waltham, MA, USA) at an excitation wavelength of 498 nm and emission wavelength of 522 nm.

Ex vivo perfusion of isolated mouse hearts and infarct size measurement. The *ex vivo* ischemia/reperfusion protocol was based on a previous study (15). A total of 18 adult male C57BL/6 mice (25-30 g; n=6 in each group) were divided into 3 different groups: An I/R group, an I/R + 400 ng/ml rhSLPI group and an I/R + 1,000 ng/ml rhSLPI group. Mice were euthanized with IP injection of pentobarbital (100 mg/kg) and heparin (150 U). Hearts were rapidly isolated and placed in ice-cold modified Krebs-Henseleit buffer (KHB) consisting of 118.5 mM NaCl, 25.0 mM $NaHCO_3$, 4.75 mM KCl, 1.18 mmol KH_2PO_4, 1.19 mM $MgSO_4$, 11.0 mM D-glucose and 1.4 mM $CaCl_2$. The excised heart was cannulated via the aorta and retrograde perfusion on a Langendorff system was performed at a constant pressure of 80 mmHg with KHB equilibrated with 95% O_2 and 5% CO_2 at 37˚C. Hearts were perfused with KHB for 10 min and were then perfused with KHB solution containing 400 ng/ml or 1,000 ng/ml rhSLPI for 30 min. Hearts from mice in the I/R group underwent perfusion with KHB alone. Hearts were then subjected to ischemia by attenuating perfusion for 30 min (no-flow). Reperfusion was performed with KHB for a further 2 h. At the end of the protocol, hearts were perfused with 5 ml 1% triphenyltetrazolium chloride (TTC) in PBS for 1 min at 37˚C and incubated in 1% TTC at 37˚C for 10 min. Atria were removed and hearts were blotted dry, weighed and stored at -20˚C for 1 week. Prior to heart tissue sectioning, the hearts were thawed, placed in 2.5% glutaraldehyde for 1 min at room temperature and embedded in 5% agarose. Agarose heart blocks were sectioned from apex to base in 0.75-mm slices using a vibratome (Agar Scientific Ltd., Essex, UK). Following sectioning, slices were fixed in 10% formaldehyde overnight at room temperature prior to incubation with PBS for 1 day at 4˚C. Sections were then compressed between glass plates (0.75-mm apart) and scanned by a Gel Doc™ XR+ System (Bio-Rad Laboratories, Inc.). All analyses of infarct size were performed by two investigators that were blinded with regards to the group assignments.

Measurement of p38 MAPK and Akt activation by western blotting. ARVMs were washed twice in ice-cold PBS prior to the addition of 200 μl 2x SDS-sample buffer containing β-mercaptoethanol. Cells were scraped and samples were transferred to pre-cooled micro-centrifuge tubes. For protein extraction, hearts from the I/R, I/R + 400 ng/ml rhSLPI group and I/R + 1,000 ng/ml rhSLPI groups (each, n=3) were perfused on a Langendorff perfusion system, subjected to 30 min stabilization, perfused with KHB containing 400 ng/ml and 1,000 ng/ml rhSLPI or KHB alone as a vehicle for 30 min prior to 10 min without perfusion (global ischemia). At the end of the global ischemia, hearts were rapidly snap frozen. Approximately 50 mg of heart samples were weighed and homogenized in 500 μl homogenization buffer (20 mM Tris pH 6.8, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and 1 cOmplete™, Mini Protease Inhibitor Cocktail Tablet; Sigma-Aldrich; Merck KGaA). Heart homogenates were centrifuged at 13,148 x g for 10 min at 4˚C. Supernatants were collected and an equal volume of 2X SDS-PAGE sample buffer containing 10% (v/v) β-mercaptoethanol and bromophenol blue dye was added. Samples were boiled for 10 min and stored at -80˚C prior to analysis. Extracted proteins (30 μg/lane) were separated on 12% SDS-polyacrylamide gels (prepared from a 30% polyacrylamide solution) and transferred to PVDF membranes that were blocked for 1 h with 5% non-fat milk and 1% BSA in Tris-buffered saline (pH 7.4) containing 0.1% Triton X-100. Membranes were then probed overnight at 4˚C with primary antibodies against total p38, diphospho-p38, total Akt and p-Akt (all 1:1,000). Following washing and exposure for 1 h at room temperature to horseradish peroxidase-conjugated secondary antibodies, antibody-antigen complexes were visualized using ECL, revealed as bands corresponding to the detected proteins of interest. Band densities were detected by

Gel DocXR+ Imaging System, quantified using Image Lab™ software (version 6.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and compared, providing information on the relative abundance of the protein of interest.

Statistical analysis. All values are presented as the mean ± standard error of the mean. All comparisons were assessed for significance using one-way analysis of variance, followed by the Tukey-Kramer test. Statistical tests were performed using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

rhSLPI-treatment prior to or during sI/R attenuates cell death following sI/R injury. The results demonstrated that rhSLPI-treatment administered 2 h prior to sI or during sI significantly reduced sI/R-induced cell death. Pretreatment of ARVMs with 1, 10, 100 and 1,000 ng/ml rhSLPI significantly increased cell viability compared with the untreated sI/R group (P<0.05; Fig. 1A). Furthermore, pre-treatment with 100 and 1,000 ng/ml rhSLPI significantly decreased LDH activity compared with the untreated sI/R group (P<0.05; Fig. 1B). In addition, treatment of ARVMs with rhSLPI during sI also significantly decreased the cell death induced by sI/R. Treatment with 100 ng/ml and 1,000 ng/ml rhSLPI significantly increased cell viability compared with the untreated sI/R group (P<0.05; Fig. 1C). Furthermore, 1,000 ng/ml rhSLPI significantly decreased LDH activity in the supernatant (P<0.05; Fig. 1D).

By contrast, rhSLPI-treatment at the onset of reperfusion (Fig. 1E) did not increase the viability of cardiac cells following sI/R. Similarly, LDH activity was not significantly reduced when cells were treated with rhSLPI at the onset of reperfusion (Fig. 1F). This was the case with all doses of rhSLPI. The results of these indicated that the rhSLPI minimum concentration that provided maximal cardioprotection in ARVMs was 100 ng/ml in case of pretreatment and 1,000 ng/ml for treatment during sI.

Identification of the maximum rhSLPI concentration required to provide cardioprotection in ARVMs subjected to sI/R. To identify the maximum rhSLPI concentration required to induce cardioprotection in ARVMs without inducing any toxicity, ARVMs were treated with 0, 200, 400, 600, 800 and 1,000 ng/ml rhSLPI 2 h prior to sI or immediately following the onset of sI. Following treatment, all groups were subjected to sI for 20 min followed by 2 h reperfusion. Cell viability and cell injury were determined.

The results indicated that following 2 h reperfusion, the percentage of viable cells in the groups treated with rhSLPI either 2 h prior to sI or at the onset of sI increased compared with the sI group. Pretreatment of ARVMs with 200, 400, 600, 800 and 1,000 ng/ml rhSLPI significantly increased the viability of ARVMs exposed to sI/R injury compared with the sI group (P<0.05; Fig. 2A). Furthermore, these concentrations significantly decreased LDH activity in the supernatants compared with the sI group (P<0.05; Fig. 2B). In addition, treatment of ARVMs with 400, 800 and 1,000 ng/ml at the onset of sI significantly increased the viability of ARVMs compared with the sI group (P<0.05; Fig. 2C) and these concentrations significantly decreased LDH activity compared with the sI group (P<0.05; Fig. 2D).

The toxicity of rhSLPI was determined by culturing ARVMs with 0, 200, 400, 600, 800 and 1,000 ng/ml rhSLPI for 24 h and then measuring cell viability and cell injury. The results indicated that treatment with all concentrations of rhSLPI did not reduce cell viability (Fig. 2E) and did not increase LDH activity (Fig. 2F). This demonstrates that rhSLPI does not induce toxicity in ARVMs.

Treatment of ARVMs by rhSLPI decreases ROS levels. To further investigate the cardio-protective effects of rhSLPI treatment during sI/R injury, intracellular ROS generation was measured following H_2O_2 challenge.

The results demonstrated that exposure to H_2O_2 significantly increased intracellular ROS levels compared with the control group (P<0.05; Fig. 3). However, treatment of ARVMs with 400, 600, 800 and 1,000 ng/ml rhSLPI significantly reduced intracellular ROS levels compared with untreated ARVMs (P<0.05). Notably, 400 ng/ml rhSLPI was the lowest concentration of rhSLPI that significantly reduced ROS production.

Treatment of ARVMs by rhSLPI attenuates p38 MAPK phosphorylation and increases Akt activation following sI. To determine the cellular signaling in response to rhSLPI treatment in ARVMs during sI/R injury, western blot analysis was performed to determine the phosphorylation of p38 MAPK and Akt.

The results demonstrated that the phosphorylation of p38 MAPK was significantly increased following sI (P<0.05; Fig. 4A). However, pretreatment with 400 and 600 ng/ml rhSLPI significantly reduced p38 MAPK phosphorylation (P<0.05). In addition, the results demonstrated that treatment with 200-1,000 ng/ml rhSLPI prior to sI significantly increased Akt phosphorylation compared with the sI group (P<0.05; Fig. 4B).

In addition, treatment of ARVMs with rhSLPI at the onset of sI reduced the phosphorylation of p38 MAPK in a dose-dependent manner (Fig. 5A). Treatment of ARVMs with 1,000 ng/ml rhSLPI at the onset of sI significantly reduced p38 MAPK activation compared with the sI group (P<0.05). The results also indicated that treatment of ARVMs with 200-1,000 ng/ml rhSLPI at the onset of sI significantly increased the phosphorylation of Akt compared with the sI group (P<0.05; Fig. 5B). This indicates that treatment of ARVMs with rhSLPI increases Akt phosphorylation and attenuates the activation of p38 MAPK.

Evaluation of the cardioprotective effects of rhSLPI in an ex vivo murine model of myocardial I/R. Hearts isolated from adult male C57BL/6 mice were perfused on a Langendorff system with buffered solutions containing 400 or 1,000 ng/ml rhSLPI during I/R injury (Fig. 6A).

The results indicate that following 30 min global ischemia and 2 h reperfusion, the infarct size was 48.40±4.23% (Fig. 6B). However, pretreatment with either 400 or 1,000 ng/ml rhSLPI significantly reduced the infarct size compared with the control (P<0.05; Fig. 6B).

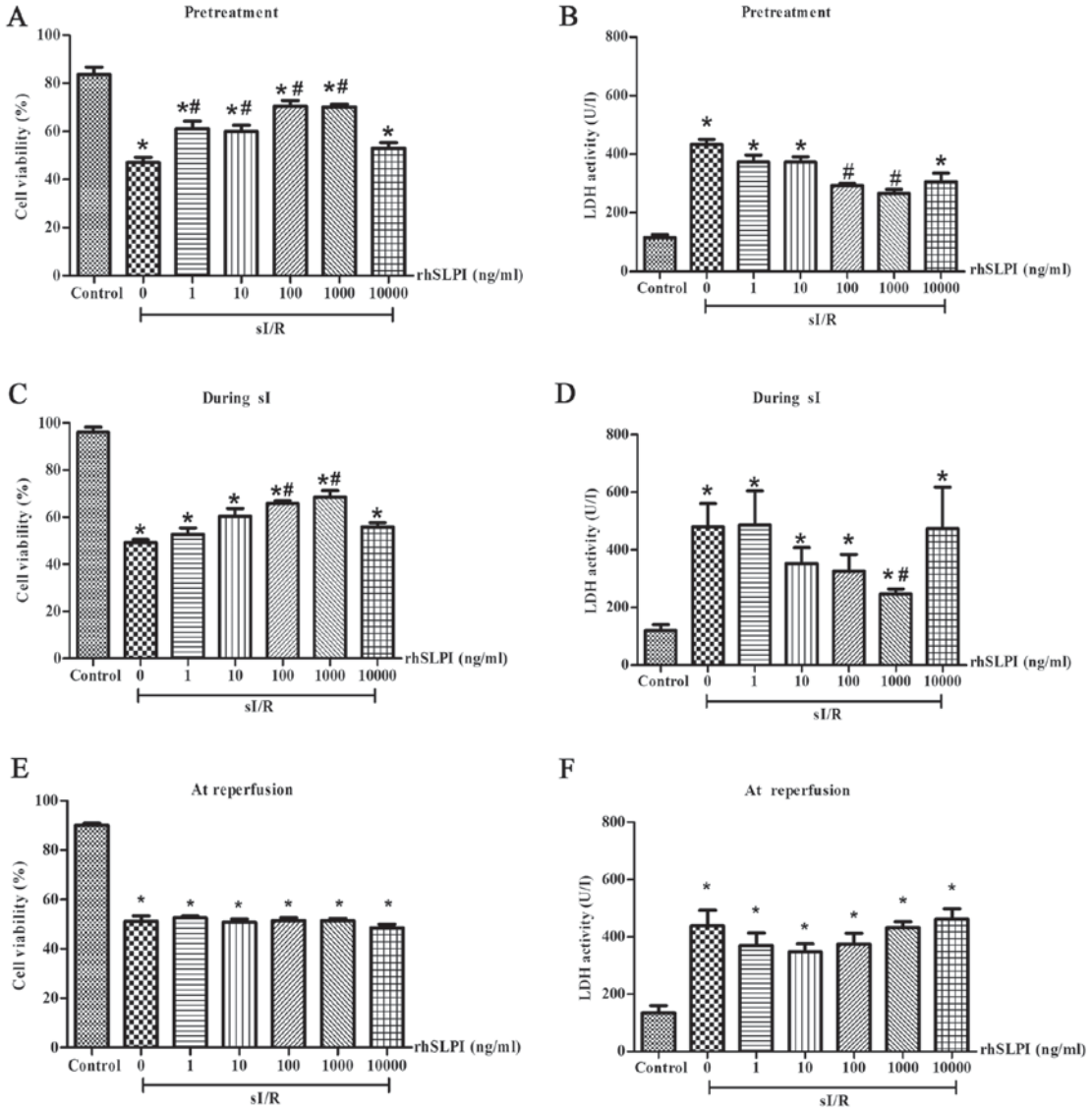

Figure 1. Optimization of the cardioprotective dose of rhSLPI for ARVMs subjected to sI/R. ARVMs were cultured with various concentrations of rhSLPI and treated over three different periods, including (A and B) 2 h prior to sI, (C and D) at the onset of sI and (E and F) at the onset of reperfusion. Following rhSLPI treatment, all groups were subjected to 20 min sI followed by 2 h reperfusion. The percentage of viable cells and released LDH activity in all groups were determined. The results are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. control group; #P<0.05 vs. sI group. rhSLPI, recombinant human secretory leukocyte protease inhibitor; sI, stimulated ischemia; ARVM, adult rat ventricular myocytes; LDH, lactate dehydrogenase; sI/R, stimulated ischemia/reperfusion.

Pretreatment with rhSLPI attenuates p38 MAPK activation and increases Akt activation in ex vivo I/R hearts. To further investigate the cardioprotective effects of rhSLPI pretreatment on *ex vivo* mouse hearts, cellular signaling in response to rhSLPI treatment following I/R injury was investigated. Hearts were perfused with 400 or 1,000 ng/ml rhSLPI for 30 min prior to global ischemia and 10 min no-flow. p38 MAPK and Akt phosphorylation ratios were determined by western blotting. The results demonstrated that, following 10 min global ischemia, p38 MAPK was highly phosphorylated in the I/R control group (Fig. 7A). However, pre-treatment with 400 or 1,000 ng/ml rhSLPI significantly decreased p38 MAPK activation in a dose-dependent manner (P<0.05; Fig. 7A). By contrast, pretreatment with 400 or 1,000 ng/ml rhSLPI significantly increased Akt phosphorylation compared with the I/R non-treated control group (P<0.05; Fig. 7B). These results indicate that pretreatment with rhSLPI increases Akt phosphorylation and attenuates p38 MAPK activation in an *ex vivo* I/R murine model.

Discussion

The present study demonstrated that rhSLPI exhibits a protective effects on *in vitro* and *ex vivo* sI/R murine models and determined the mechanism by which treatment with rhSLPI inhibits the phosphorylation of p38 MAPK and activates the Akt cell survival pathway in ARVMs and the *ex vivo* heart. The results of the current study indicated that in the case of ARVMs that underwent sI/R injury, the administration of exogenous rhSLPI, prior to or at the onset of sI significantly reduced cardiac cell death and cell injury.

During myocardial I/R injury, levels of pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin (IL) 6, IL-8, and mitochondrial pyruvate

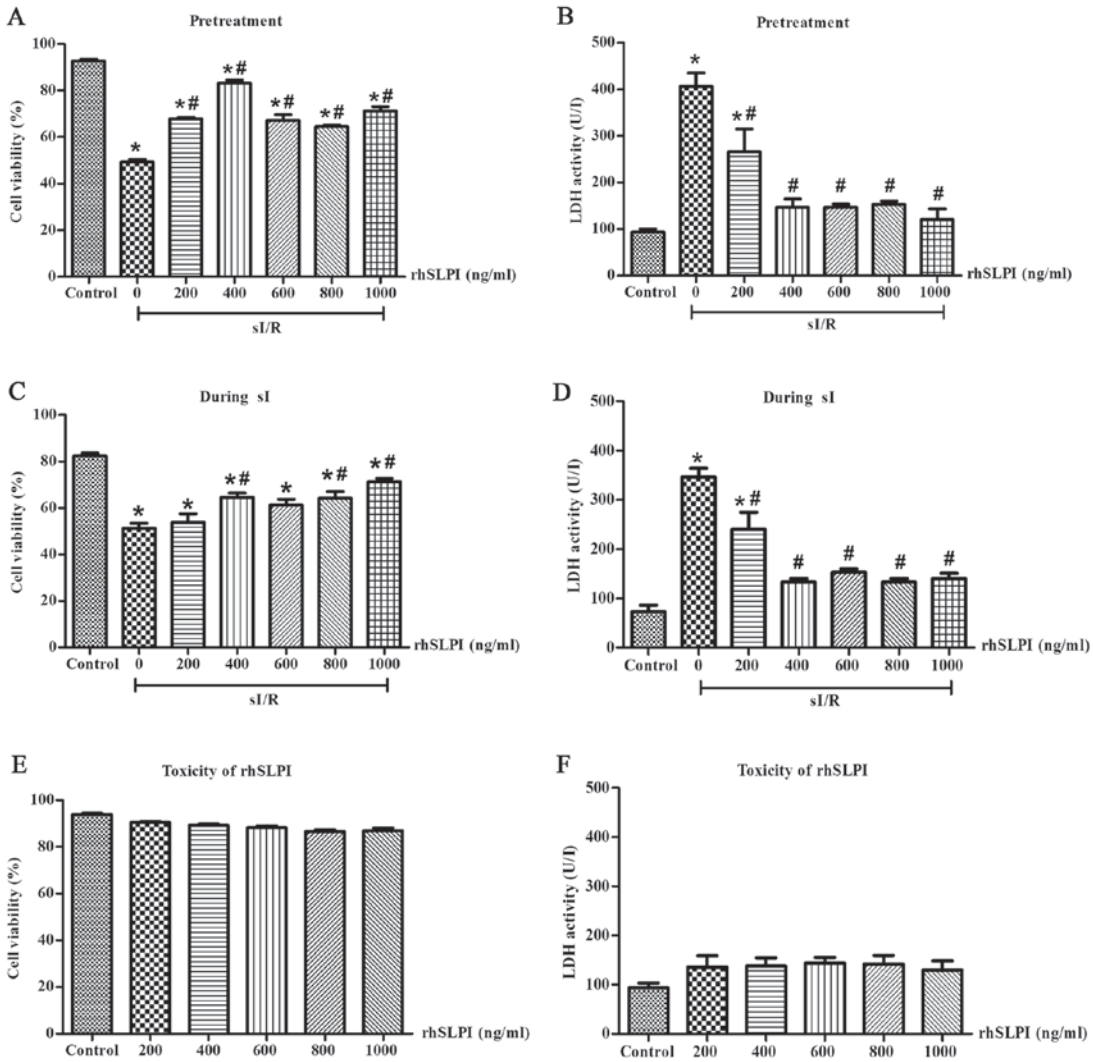

Figure 2. Determination of the maximum rhSLPI concentration required to provide cardioprotection in ARVMs subjected to sI/R. The percentage of (A) viable cells and (B) LDH activity following pretreatment of ARVMs with various concentrations of rhSLPI. The percentage of (C) viable cells and (D) LDH activity following treatment of ARVMs with various concentrations of rhSLPI during sI. The percentage of (E) viable cells and (F) LDH activity following treatment of ARVMs with various concentrations of rhSLPI without sI. The results are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. control group; #P<0.05 vs. sI group. rhSLPI, recombinant human secretory leukocyte protease inhibitor; ARVM, adult rat ventricular myocytes; sI, stimulated ischemia; sI/R, stimulated ischemia/reperfusion; LDH, lactate dehydrogenase.

carrier-1 (MPC1), are upregulated and secreted from adjacent cardiomyocytes, resulting in leukocyte infiltration (5-7). Infiltrated leukocytes, particularly neutrophil, secrete numerous serine protease enzymes, including cathepsin G, elastase and trypsin, which contribute to heart tissue damage, myocardial necrosis and functional impairment (5,7). Therefore, inhibiting protease activity, as well as attenuating inflammatory responses, may be a promising therapeutic strategy to prevent and treat cardiac tissue injury.

Previous studies have determined the effects of protease inhibitors on ischemic injury. In a rabbit model of heterotopic cardiac transplantation, the serine elastase inhibitor elafin decreased post-cardiac transplant coronary arteriopathy and decreased myocardial necrosis following transplantation (16). Treatment with two specific elastase inhibitors, elafin and ICI 200880, in *in situ*-perfused rat heart models of repetitive ischemia and myocardial infarction improved regional myocardial function and reduced infarct size (17). Furthermore, inhibition of the serine protease

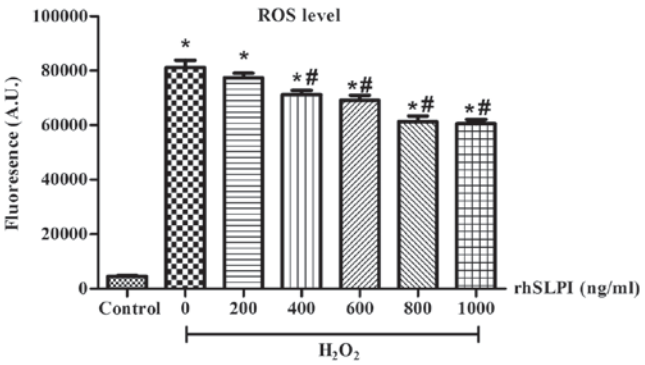

Figure 3. Cellular ROS levels following treatment of ARVMs with rhSLPI. ARVMs were incubated with carboxy-H2DCFDA and treated with 0, 200, 400, 600, 800 and 1,000 ng/ml rhSLPI. Following incubation, H_2O_2 was applied to each group and ROS production was determined using the EnSpire Multimode Plate Reader. Data are presented as the mean ± standard error of the mean (n=3). *P<0.05 vs. control group; #P<0.05 vs. H_2O_2 treated group. ROS, reactive oxygen species; ARVM, adult rat ventricular myocytes; rhSLPI, recombinant human secretory leukocyte protease inhibitor; A.U., arbitrary unit.

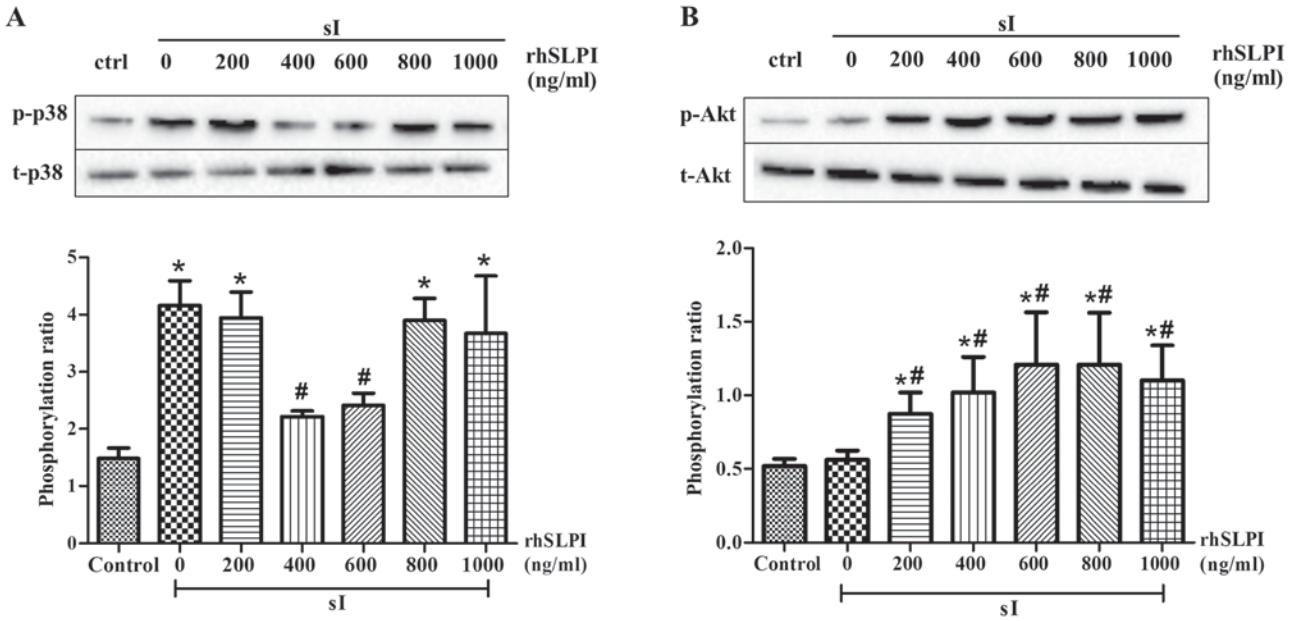

Figure 4. Cellular signaling in response to pretreatment of ARVMs with rhSLPI, subjected to ischemia/reperfusion. ARVMs were treated with 200, 400, 600, 800 and 1,000 ng/ml rhSLPI for 2 h prior to sI. The phosphorylation of (A) p38 MAPK and (B) Akt was determined by western blot analysis. Each bar graph represents the phosphorylation ratio (p-/t-) of p38 MAPK and Akt. *P<0.05 vs. control group; #P<0.05 vs. sI group. ARVM, adult rat ventricular myocytes; rhSLPI, recombinant human secretory leukocyte protease inhibitor; sI, stimulated ischemia; MAPK, mitogen-activated protein kinase; p-phosphorylated; t-, total.

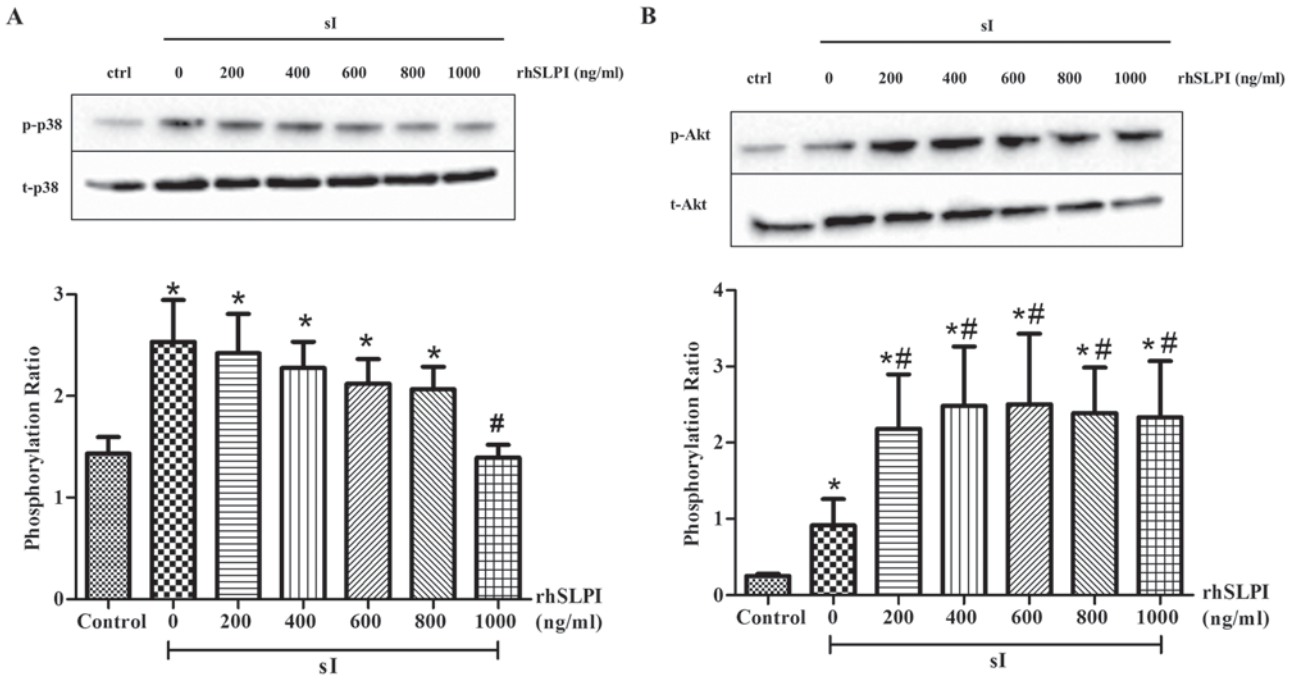

Figure 5. Cellular signaling following treatment of ARVMs with rhSLPI during sI. ARVMs were treated with 200, 400, 600, 800 and 1,000 ng/ml rhSLPI at the onset of sI. The activation of (A) p38 MAPK and (B) Akt was determined by western blot analysis. Each bar graph represents the phosphorylation ratio (p-/t-) of p38 MAPK and Akt. *P<0.05 vs. control group; #P<0.05 vs. sI group. ARVM, adult rat ventricular myocytes; rhSLPI, recombinant human secretory leukocyte protease inhibitor; sI, stimulated ischemia; MAPK, mitogen-activated protein kinase; p-, phosphorylated; t-, total.

enzymes secreted by neutrophils exhibited cardioprotective effects against myocardial I/R injury (18,19). Treatment with PR-39, a potent neutrophil inhibitor, in a murine model of myocardial I/R injury significantly inhibited the recruitment of leukocytes into inflamed tissue, thus decreasing infarct size (18). In a canine model of heart transplantation, treatment with the neutrophil elastase inhibitor ONO-5046 Na reduced I/R injury and inhibited neutrophil elastase and inflammatory cytokine release (19). However, SLPI may be the broadest and most promising method of treating myocardial I/R injury compared with other protease inhibitors because SLPI can inhibit various protease enzyme and exhibits anti-oxidant functions (20). SLPI attenuated cell injury in a murine hepatic I/R model by inhibiting neutrophil accumulation (21).

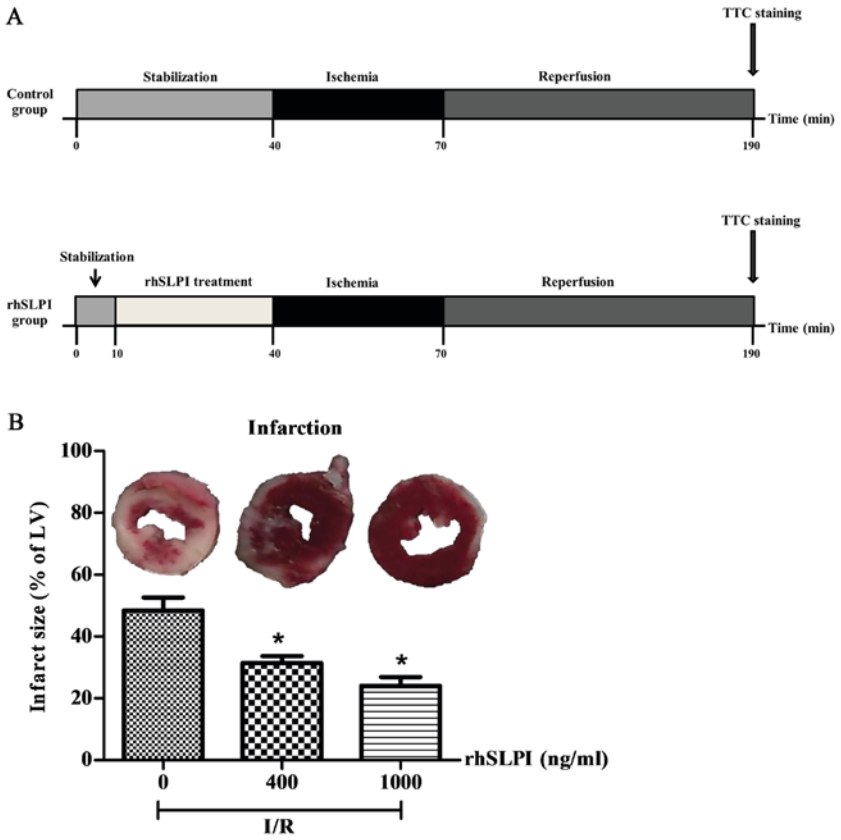

Figure 6. The effect of rhSLPI treatment on the myocardial infarct size of *ex vivo* murine hearts during myocardial I/R. (A) The study protocol for all experimental groups. The hearts of adult male C57BL/6 mice were perfused for 40 min with modified Krebs solution, or with modified Krebs solution containing 400 ng/ml rhSLPI or 1,000 ng/ml rhSLPI. Hearts were then subjected to 30 min global ischemia and 2 h reperfusion. (B) Infarct size was determined by TTC staining. Each bar graph represents the mean ± standard error of the mean (n=6). *P<0.05 vs. I/R alone. rhSLPI, recombinant human secretory leukocyte protease inhibitor; I/R, ischemia/reperfusion; LV, left ventricle.

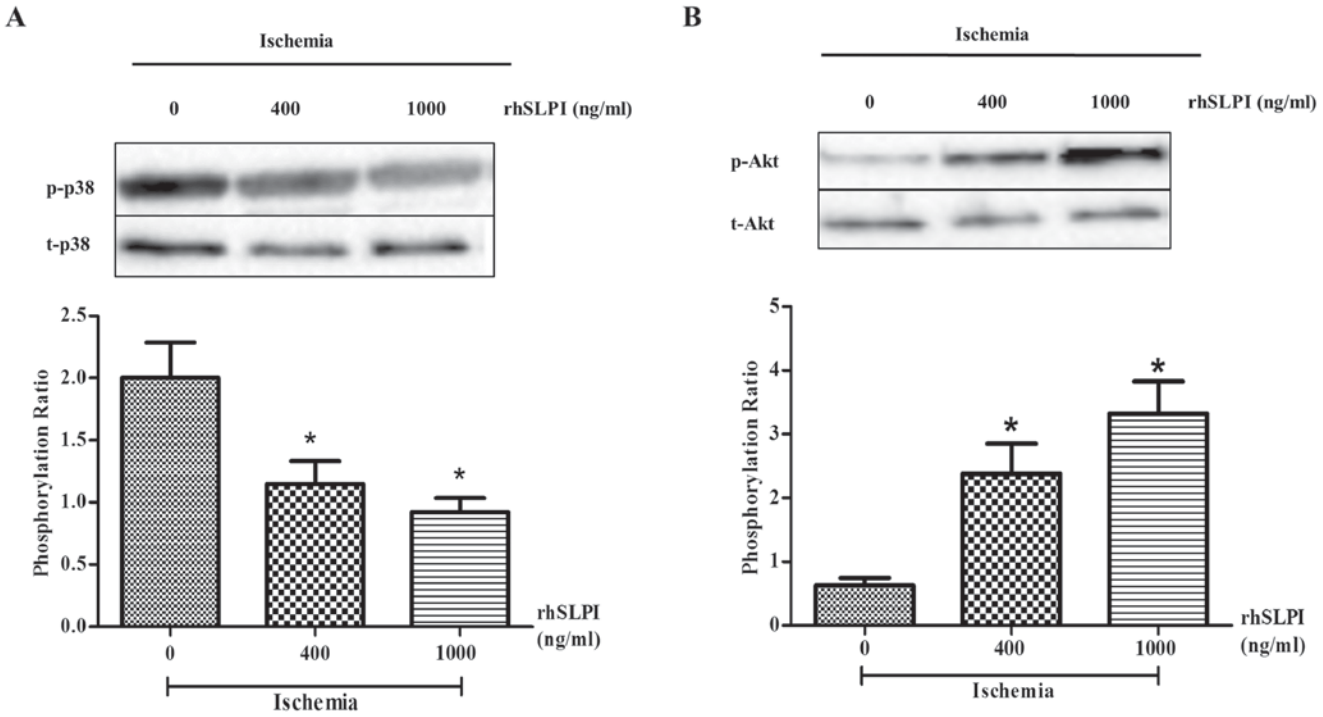

Figure 7. Signaling in response to treatment of *ex vivo* murine hearts with rhSLPI during myocardial I/R. The hearts of adult male C57BL/6 mice were perfused for 40 min with modified Krebs solution or modified Krebs solution containing 400 ng/ml or 1,000 ng/ml rhSLPI followed by 10 min global ischemia. Hearts were homogenized with homogenizing buffer and protein was collected. The activation of (A) p38 MAPK and (B) Akt was assessed using western blot analysis. Each bar graph represents the fold phosphorylation of p38 MAPK and Akt. *P<0.05 vs. I/R control group (n=3). rhSLPI, recombinant human secretory leukocyte protease inhibitor; MAPK, mitogen-activated protein kinase; p-, phosphorylated; t-, total; I/R, ischemia/reperfusion.

Furthermore, SLPI decreased serum levels of TNF-α, the CXC chemokine macrophage inflammatory protein, and suppressed activation of the transcription factor nuclear factor (NF)-κB in the liver (21). Exogenous rhSLPI added to cold-preservative buffer improved the cardiac score of hearts (a system that grades myocardial contraction) following transplantation and reduced the expression of protease enzymes and pro-inflammatory cytokines in mice undergoing heart transplants (13).

Studies have investigated the effects of SLPI on inflammation. SLPI is a protein involved in downregulating macrophage responses against bacterial lipopolysaccharides (LPS) by inhibiting NF-κB activation (22). Ding *et al* (23) demonstrated that the anti-inflammatory effects of SLPI on macrophages may be due to the inhibition of cluster of differentiation (CD)14 on the macrophage membrane or by inhibiting the formation of LPS-soluble CD14 complexes. It has been demonstrated that exogenous SLPI is internalized into monocytes and is distributed throughout the nucleus and cytoplasm. It then inhibits NF-κB activation by blocking the degradation of the inhibitor of NF-κB (24). Interestingly, Taggart *et al* (24) demonstrated that SLPI competes with the NF-κB subunit p65 to bind to the promoter of NF-κB, resulting in the decreased production of pro-inflammatory cytokines. Furthermore, the spraying of SLPI aerosols suppresses respiratory epithelial neutrophil elastase (NE) levels and attenuates the inflammation of the cystic fibrosis epithelial surface by reducing IL-8 levels (25). Adapala *et al (*26) reported that SLPI expression in the adipose tissue is upregulated in obesity and that the pretreatment of adipocytes with exogenous SLPI results in the downregulation of LPS-induced IL-6 gene expression and protein secretion in adipocytes. In addition, exogenous SLPI increased the proliferation and differentiation of adult neural stem cells via the upregulation of cyclin D1 and suppression of the cell differentiation regulator HES1 (27). The treatment of monocytes with exogenous SLPI inhibited the TNF-α-induced caspase-3 activation and the DNA degradation associated with apoptosis in monocytes (28). The results of other studies investigating different types of cells suggest that treatment with rhSLPI induces similar anti-inflammatory and anti-apoptotic effects (29-31).

The excessive formation of ROS during I/R injury induces cardiac cell death directly by inducing cell membrane and protein damage (32,33) or indirectly by activating pro-apoptotic pathways, as well as recruiting inflammatory cells (32,34-38). It has been suggested that SLPI reduces cellular ROS generation. The administration of rhSLPI suppressed NE and glutathione levels in the respiratory epithelial lining fluid of the lung and caused a concomitant increase in anti-H_2O_2 capacity (39). Furthermore, the overexpression of rhSLPI and glutathione peroxidase-3 reduced oxidant-induced lung injury and inflammation (40). This indicates that administration of rhSLPI may be used to treat diseases similar to myocardial I/R injury that are characterized by an excess of serine proteases and oxidative stress. The results of the current study demonstrate that treatment with ARVMs by rhSLPI decrease cellular ROS generation during H_2O_2 challenge. To the best of our knowledge, the current study is the first to demonstrate that the treatment of ARVMs with rhSLPI reduces cellular ROS generation and may therefore be an effective method of treating ARVMs in sI/R. However, the reduction of ROS production and cardiac cell death in ARVMs following treatment with rhSLPI may be due to increases in glutathione levels in cardiac cells rather than a direct effect on ROS scavenging. Therefore, further research investigating the mechanisms by which rhSLPI reduces intracellular ROS levels in I/R is warranted.

Myocardial I/R injury is mediated by numerous signaling pathways, including p38 MAPK. Myocardial I/R injury activates p38 MAPK, which induces cardiac cell death (15,41-45). The pre-clinical investigation indicated that the reduction of p38 MAPK activation reduces myocardial injury (44). Given that the treatment of ARVMs with rhSLPI reduces cell death following I/R injury, it was hypothesized that the decrease in cardiac cell death following treatment of ARVMs with rhSLPI is caused by the attenuation of p38 MAPK activation. The results of the current study indicated that the administration of exogenous rhSLPI prior to or at the onset of sI significantly attenuated the phosphorylation of p38 MAPK. These results indicate that rhSLPI administration prior to or at the onset of ischemic insult may attenuate the activation of p38 MAPK and may therefore be an effective method of protecting cardiac cells against I/R.

Akt is a serine/threonine protein kinase and serves an important role in cell survival (46-49). The activation of Akt signaling protects cardiac cells from apoptosis, resulting in the attenuation of myocardial I/R injury (46-49). It was therefore hypothesized that the treatment of ARVMs with rhSLPI may stimulate Akt phosphorylation during sI/R injury. The results demonstrated that treatment of ARVMs with rhSLPI prior to or during sI significantly activated Akt phosphorylation. This suggests that the administration of rhSLPI not only protects the cell by inhibiting the activation of p38 MAPK but also increases cell survival by activating the Akt pathway.

Although it has been demonstrated that various treatments/interventions administered during reperfusion are effective at reducing infarct size, the results of the current study indicated that administering rhSLPI at the onset of reperfusion failed to protect ARVMs from sI/R induced cell death and cell injury. These results were similar to those of previous studies investigating the effects of treatments or interventions administered prior to ischemia, which may protect the heart from I/R injury (13,15). Indeed pre-treatment with the p38 MAPK inhibitors SB203580 and BIRB796 for 30 min prior to ischemia may significantly reduce infarct size (15). The results of a previous study conducted in an ischemic rat heart model indicated that SB203580 administered either prior to or during ischemia decreased the incidence of ventricular tachycardia/ventricular fibrillation in the ischemic heart, decreased the phosphorylation of heat shock protein 27 and increased the phosphorylation of connexin 43 (50) However, treatment with SB203580 at the onset of reperfusion failed to protect the heart from ischemic insult (50). This may be due to the fact that, when administered at reperfusion, SB203580 does not inhibit the activation of p38 MAPK and the pro-apoptotic protein Bax, and does not protect the cardiac mitochondrial membrane potential (45). In addition, previous studies demonstrated that pretreatment with various types of compounds (including amifostine, barbaloin, simvastatin, lysophosphatidic acid and icariin) may also exhibit cardioprotective effects against I/R injury (51-55), similar to the results of the current study. Therefore, the results of the current study

suggest that treatment with rhSLPI (400-1,000 ng/ml) prior to or at the onset of ischemia is most effective at protecting cardiac cells and reducing cardiac cell injury following I/R. Pre-treatment with 1,000 ng/ml rhSLPI in *ex vivo* murine hearts significantly reduced the infarct size. These results indicate that timing SLPI treatment with respect to the onset of ischemia is an important determinant of its therapeutic efficacy.

There were a number of limitations of the current study. Treatment of ARVMs by rhSLPI or treatment of *ex vivo* hearts with rhSLPI may not be representative of real-world physiological settings. The *ex vivo* model is useful in determining the actual effect of drugs or substance testing, as the heart undergoes direct perfusion with the test substance and the effect serum proteins binding to the test substance is avoided. In addition, the global ischemia protocol used in the current study is well-suited to study the effects of ischemia and hypoxia and determine the release of cellular constituents from coronary effluent, including enzymes and proteins, as well as markers of cardiac metabolism (56). The protocols used in the current study, including perfusion setting, fixation of the heart tissue by glutaraldehyde or formalin and determination of the infarct size are standard methods that have been used in previous studies (14,15,50,57,58). As aforementioned, an *ex vivo* model may not be representative of real clinical settings; therefore other relevant models, including treatment of intact hearts in an *in vivo* model of I/R may produce more relevant functional data that are closely associated with real physiological events in the heart, allowing a more reliable interpretation of the results. In addition, the number of animals used in each experiment in the current study was small; therefore a larger sample size should be used in future *in vivo* or clinical studies.

In conclusion, the results of the current study indicate that treatment with rhSLPI exhibits cardioprotective effects against myocardial I/R injury in *in vitro* and *ex vivo* models by reducing intracellular ROS levels and infarct size, attenuating p38 MAPK phosphorylation and activating Akt phosphorylation. The results suggest that rhSLPI may be developed as a potential therapeutic strategy to treat patients with ischemic heart disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The present study was approved by the animal ethics committee of the Center for Animal Research, Naresuan University (approval no. NU AE550732).

Authors' contributions

EP and SK conceived and designed the experiments. EP, JS, SBL, JN, HN, MM and SK performed the experiments and analyzed the data. EP, MM and SK wrote, re-checked, and proofread the paper.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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