THE EFFECT OF RECOMBINANT HUMAN SECRETORY LEUKOCYTES PROTEASE INHIBITOR (rhSLPI) TREATMENT ON CARDIAC FIBROBLAST SUBJECTED TO ISCHEMIA/REPERFUSION INJURY

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Abstract

Myocardial ischemia (MI) is the leading cause of death in people around the world. Although, myocardial reperfusion has been shown to be the most effective therapeutic way, but reperfusion seems to be double-edged sword. Myocardial ischemia/reperfusion injury(I/R) is a condition cause by infiltration of neutrophils and overproduction of ROS, which has been known to activate p38 MAPK. This could aggravate cardiac cell injury and death. In heart compose of cardiomyocytes and non-cardiomyocytes as cardiac fibroblast. The main function of cardiac fibroblast is maintaining formation structure of the heart. The pharmacological protease inhibitor, recombinant human Secretory Leukocytes Protease Inhibitor(rhSLPI) has been reported could reduced cardiac cell injury, cell death, infarct size and improve cardiac function. However, the effect of an *in vitro* experiment of rhSLPI treatment in cardiac fibroblasts subjected to simulated ischemia/reperfusion have not been studied. The objectives of this study were to determine the effect of recombinant human Secretory Leukocyte Protease Inhibitor (rhSLPI), by mean of treatment on simulated ischemia/reperfusion injury and its cellular mechanisms. The adult rat cardiac fibroblasts were isolated from adult male Wistar rats by Langendorff perfusion-collagenase based technique. The cells were pretreatment with various concentrations of rhSLPI. Then, the cells were subjected to ischemia/reperfusion. The cell viability was determined by MTT assays. The cellular ROS production was measured by DCFH-DA assays. In addition, the cellular protein extract was collected and subjected to SDS-PAGE, followed by determine p38 MAPK with western blotting analysis. The result showed that the pretreatment of rhSLPI with 1 μg/ml could reduce cell death and cellular ROS production. Moreover, pretreatment of rhSLPI could reduce cell death by down regulating p38 MAPK. In conclusion, the pretreatment of rhSLPI in adult rat cardiac fibroblast exhibit cardioprotective effects in I/R injury by reducing cell death against I/R injury, reduced intracellular ROS production and attenuated p38 MAPK activation.

Keywords: Cardiac fibroblast, rhSLPI, Myocardial ischemia/reperfusion injury, p38 MAPK, Cardioprotective effects, reactive oxygen species (ROS)

Introduction

Myocardial ischemia cause of cardiac cell injury and cell death by blockade coronary arteries cannot receive enough blood flow and oxygen (Turer, & Hill, 2010, p.360-368; Hausenloy, & Yellon, 2013, p.92-100). The reperfusion seems to help the heart from myocardial ischemia, but not reduce cell death from reactive oxygen species (ROS) (Braunwald, 1998, p.1771-1774; See, Kompa, & Krum, 2004, p.149-154). Moreover, during

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myocardial ischemia, the leukocytes as neutrophil could release ROS and secrete many serine protease enzymes e.g. collagenase, elastase (Jordan, et al., 1999, p.860-876; Kaiser, et al., 2004, p.15524-15530). Additionally, activation of the 38kDa mitogen activated protein kinase (p38 MAPK), which plays an important role in cellular responses to external stress signaling during I/R injury, aggravates cell injury and cell death (Kaiser, et al., 2005, p.H2747-H2451; Kumphune, & Chattipakorn, 2012, p.513-524; See, et al., 2004, p.1679-1689). Therefore, reduction of I/R injury and inhibition of the serine protease activity and ROS could reduce cell death, improve the heart function in I/R injury. Interestingly, the protease enzyme locates in epithelial cells lining mucous membrane of productive, respiratory and intestinal tracks called Secretory Leukocytes Protease Inhibitor (SLPI), these protease enzymes inhibitor could inhibit the serine protease activity and decrease inflammatory cytokines such as NF-kB, TNF-β (Doumas, Kolokotronis, & Stetanopoulos, 2005, p.1271-1274; Morean, et al., 2008, p.284-295). Recent evidence showed that the effect of recombinant human SLPI could reduce cell injury, cell death, ROS production and infarct size (Schneeberger, et al., 2008, p.773-783; Prompunt, 2016) Therefore, any strategies implementing the SLPI treatment in ischemic heart mean to save life. Although SLPI seems to benefit in protecting the heart from injury, the underlying mechanisms have not been clarified. In fact, the heart consists of more than one population, there are cardiomyocytes and non-cardiomyocytes (Brilla, Maisch, & Weber, 1993, p.57-61). The main population of non-cardiomyocytes is cardiac fibroblast, cardiac fibroblast maintains formation structure of the heart and directly impacts cardiomyocytes function via cell-cell interaction (Torre-Amione, et al., 1996, p.1201-1206; Bowers, McFadden, Borg, & Baudino, 2012, p.107-114). Accordingly, the I/R injury cause of the heart cell death, that could induce cardiac fibroblast death and left myocardial fibrosis (Kawaguchi, et al., 2011, p.594-604; Chen, & Frangogiannis, 2013, p.945-953). So, if any substances containing cardioprotective effect, e.g. SLPI, these protease enzymes inhibitor could be possibly protecting cardiomyocytes during I/R injury. In addition, the protective effects of SLPI expression in cardiac fibroblast itself still have not been intensively investigated. Therefore, the aim of this study is to investigate the effect and underlying mechanism of SLPI treatment to protect cardiac fibroblast on ischemia/reperfusion injury. The findings from this study provide useful information about effect of rhSLPI for future therapeutic approach at the clinical level.

Methodology

Cell culture

    Adult Rat Cardiac Fibroblasts (ARCFs) was isolated from adult male Wistar rat by Langendorff perfusion-collagenase based technique (Santiago, et al., 2010, p.1573-1584). The cells and was cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 5,000 units of penicillin and streptomycin. Cells were cultured at 37°C, 5% CO_2 + 95% O_2 throughout the experiments.

Determination effect of recombinant human SLPI (rhSLPI) in ARCFs on sI/R

    ARCFs were pretreatment with rhSLPI concentration at 1ng/ml, 10ng/ml, 100ng/ml, 1 μg/ml and 10 μg/ml for 2 h, cells were then subjected to simulated ischemia for 40 min and followed by reperfusion 24 h and for measuring cell viability, cellular ROS production and expression of p38 MAPK.

Measurement of cell viability

The measurement of cell viability was performed by MTT cell survival assay based on the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in the presence of mitochondrial reductases. At the end of reperfusion period, cells were incubated with 0.01g/ml MTT solution for 2 h at 37°C. After that, dimethyl sulfoxide (DMSO) was used for dissolving the converted dye. The optical density was determined spectrophotometrically at λ 490 nm. The percentage of cell viability was calculated by comparing the optical density of treated samples with untreated control group (100% viability).

Simulated Ischemia/Reperfusion

The simulated Ischemia/Reperfusion was performed by "Chemical-Ischemia" based on metabolic blockade, which used compounds as deoxy-glucose; alteration ATP production by glycolysis, sodium dithionite; oxygen scavenger and sodium lactate; cellular acidosis. The ARCFs were pretreatment with rhSLPI concentration at 1ng/ml, 10ng/ml, 100ng/ml, 1 μg/ml and 10 μg/ml for 2 h. Then, simulated ischemia was induced by incubate ARCFs with ischemic buffer (10X stock basic buffer (137 mM NaCl, 3.58 mM KCl, 0.49 mM $MgCl_2 \bullet 6H_2O$, 1.8 mM $CaCl_2 \bullet 2H_2O$, 4 mM HEPES), sterile ddH_2O, 20 mM 2-deoxyglucose, 30% Na lactate, 1.0 mM Na dithionite) for 40 min at 37°C. After ischemia simulation, the ischemic buffer was removed and the cells were subjected to reperfusion by adding DMEM complete medium before further incubating at 37°C, 5% CO_2 for 24 h. After reperfusion, the culture medium was collected for assessing cell viability was determined by MTT assay. The percentage of cell viability was calculated same as mentioned above.

Determination of cellular ROS production level

The DCFH-DA is a non-fluorescence reagent which can be permeable to the cells and the intra-cellular esterases cleave two ester bonds in the molecule of DCFH-DA producing a non-fluorescent 2',7'-Dichlorodihydrofluorescin (H2DCF), which is cell membrane-impermeable product. The H2DCF is a non-fluorescent molecule, which accumulates in the cell and rapidly oxidized to highly fluorescent 2',7'-Dichlorodihydrofluorescein (DCF) by ROS. The ARCFs were treated with DMEM medium containing 250 μM carboxy-H2DCFDA. After that, the cells were incubated in dark chamber for 30 min at 37°C. After that, DMEM completed medium containing 0 ng/ml at 1ng/ml, 10ng/ml, 100ng/ml, 1 μg/ml and 10 μg/ml rhSLPI were added and incubated for 1 h at 37°C. After incubation, 250 μM H_2O_2 was applied. The cells were incubated in this condition for 30 min at 37°C. After incubation, the ROS activity was determined by the EnSpire Multimode Plate Readers (PerkinElmer, Massachusetts, USA). The fluorescence signal was read using excitation at wavelength 498 nm and emission wavelength at λ522 nm. The arbitrary unit (AU) of 498/522 nm was calculated by comparing the AU of treated H_2O_2+ rhSLPI samples with control group.

Determination of p38MAPK response rhSLPI by Western blotting analysis

Cells, which exposed to rhSLPI 1 μg/ml and control untreated cells, were washed twice in ice-cold PBS before addition of 200 μl of 2x SDS-sample buffer, containing 2-mercaptoethanol, per well in a 6 well plate. Cells were scraped and the samples were taken and transferred to the new pre-cooled microcentrifuge tube. The samples were boiled for 10 min. Extracted proteins were separated on 10 % SDS- polyacrylamide gel electrophoresis in the electrical field using a voltage at 90V for 10 min following by 120V for 2 h. The proteins were transferred to PVDF membranes (Hybond-P GE Heath Care) under an electric current of 15 mV for 1 h.

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Then, membranes were disassembled from the gel and incubated in blocking solution (5% (w/v) dried skimmed milk powder, in TBST) for 1 h. The membrane containing transferred protein was incubated with primary antibody against p38 MAPK, which diluted at 1: 1,000 in 1% skim milk + TBST buffer at 4°C overnight. After incubation, the membranes were washed for 5 min occasions before exposing to the horseradish peroxidase (HRP) conjugated secondary antibody diluted at 1: 1,000 in 1% skim milk + TBST for 1 h at room temperature. Following four further washes for 5 min in TBST, membranes were developed using an enhanced chemiluminescence (MERK) technique. Bands corresponding to the detected protein of interest were measured by Chemidoc^{TM} XRS (Bio-Rad) and analyzed relative abundance of the protein by image J.

Statistical Analysis

All values were expressed as Mean ± SEM. All comparisons involving more than one group were assessed for significance using one-way analysis of variance (ANOVA), followed when appropriate by the Tukey-Kramer test. The statistical tests were performed using commercially available software (Lab chart Prism version 5). A p-value less than 0.05 was considered as statistically significant.

Results and Discussion:

The exposure time of ARCFs with simulated ischemia (sI) buffer significantly reduced percentage of cell viability in tine dependent manner. The optimal condition was considered from exposure time, which gave 50% cell death and 50% cell viability (50% estimation). Hence, the exposure time at 40 min was the optimal sI time for ARCFs and was used in all sI experiments Figure 2.
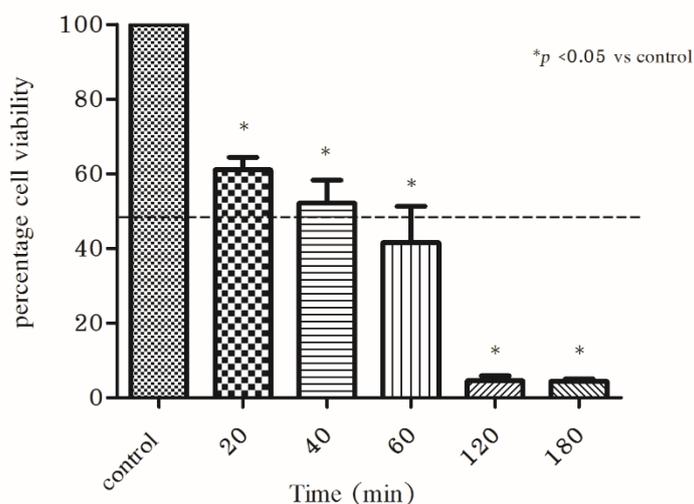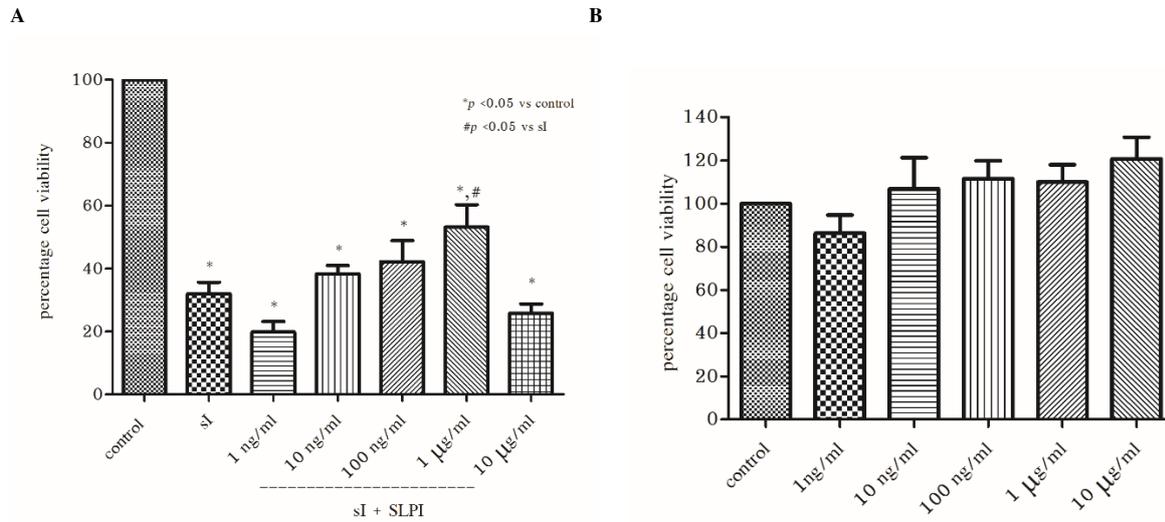

Figure 1. Optimization of simulated ischemia (sI) duration. The ARCFs were exposed with sI buffer at various periods, including 20 min, 40 min, 60 min, 120 min, and 180 min. the determination was MTT cell survival assay. Each bar graph represents mean ± S.E.M. from 6 independent experiments (ANOVA).

The effect of pretreatment with rhSLPI at 1ng/ml, 10ng/ml, 100ng/ml, 1 μg/ml and 10 μg/ml were determined in ARCFs, which subjected to sI/R. The results showed in Figure3A, pretreatment of ARCFs with rhSLPI at 1 μg/ml (53.22 ± 18.86%), significantly reduced simulated ischemia induced cell death when compare

to the sI group $(31.95 \pm 9.85\%)$. Additionally, the cellular toxicity of rhSLPI treatment on ARCFs was performed. The results showed that treatment with rhSLPI did not reduce cell viability (Figure3B)

Figure 3 Optimization the cardio-protective dose of rhSLPI treatment in ARCFs subjected to simulated ischemia/reperfusion. **A:** Percentage of cell viability in pretreatment of ARCFs with various concentrations of rhSLPI. **B:** Percentage of cell viability of treatment of ARCFs with various concentrations of rhSLPI without sI for interpreting toxicity from rhSLPI. Each bar graph represents mean ± S.E.M. from 3 independent experiments (ANOVA).

The cellular ROS production was determined in ARCFs that pretreatment with rhSLPI at 1ng/ml, 10ng/ml, 100ng/ml, 1 µg/ml and 10 µg/ml by DCFHA assay. The results showed in Figure4, the pretreatment of ARCFs with 1 µg/ml rhSLPI significantly reduced the cellular ROS level in the ARCFs after H_2O_2 challenging when compared to the untreated ARCFs $(44420 \pm 4750$ A.U. vs 58990 ± 4548 A.U, respectively$)$.
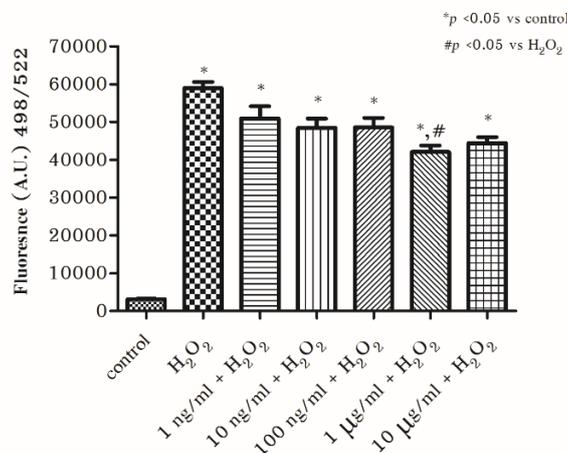
Figure 4 Determination of cellular reactive oxygen species (ROS) level after treating the ARCFs with rhSLPI. ARCFs were incubated with carboxy-H2DCFDA and then, treated with rhSLPI. After incubation, H_2O_2 was applied to each group. Each bar graph represents mean ± S.E.M. from 3 independent experiments (ANOVA).

The cellular signaling response to rhSLPI in the ARCFs during sI/R injury was determined in ARCFs and pretreatment with 1 µg/ml of rhSLPI. The protein samples were subjected to Western blotting analysis against the specific antibody for p38 MAPK. The results showed in Figure5, the pretreatment with rhSLPI at 1 µg/ml significantly reduced p38 MAPK phosphorylation.
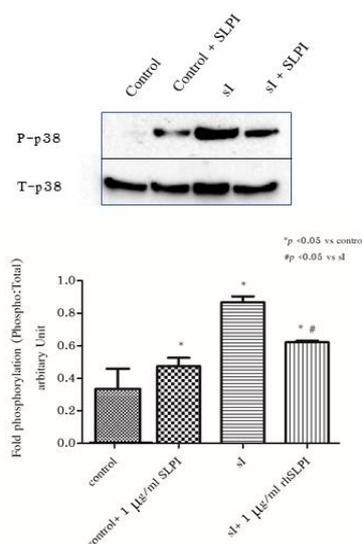
Figure 5 Effect of pretreatment 1 µg/ml of rhSLPI in ARCFs on cellular protein signaling response. The proteins were extracted and subjected to western blotting against p38 MAPK. Each bar graph represents for phosphorylation of p38 MAPK from 3 independent experiments (ANOVA).

In the previous study, rhSLPI was demonstrated the cardio-protective effect to decrease infarct size when rhSLPI was added to the preservation solution in murine cardiac transplant model (Schneeberger, et al., 2008, p.773-783) Furthermore, *in vitro* study in cardiac myoblast (H9c2) and adult rat ventricular myocyte (ARVM) showed the anti-ischemic effect of overexpression SLPI and rhSLPI treatment could reduce cell injury and cell death and reduce infarct size in murine model (Prompunt, 2016). However, the heart composes of cardiomyocytes and non-cardiomyocytes as cardiac fibroblast account for 70% and plays role as a "sentinel cell" by construct scaffold to hold other cells together (Nag, 1980, p.41-61). Interestingly, the previous study showed that secretome of cardiac fibroblasts could reduce cardiomyocytes death in co-culture model, that was supported communication between two cell-types (Abrial, et al., 2014, p.56-65). Therefore, if rhSLPI could protect cardiac fibroblast from I/R injury, it could possibly also protect the heart from I/R injury as well as post-infarction remodeling. In this study, the effect of rhSLPI treatment in various concentration on I/R injury was investigated in primary culture of isolated adult rat cardiac fibroblast or ARCFs. We hypothesized that treatment of rhSLPI could protect ARCFs similar to previous studies that were mentioned above.

Pretreatment of rhSLPI on ARCFs for 2 h could protect the ARCFs from sI/R injury induced cell death. The results showed that rhSLPI at 1 µg/ml gave protective effect with the highest percentage of cell viability. This

result was consistent with previous study in ARVMs model (Prompunt, 2016). However, at 10 µg/ml of rhSLPI reduced percentage cell viability when compared with 1 µg/ml, we hypothesized that the high concentration of rhSLPI cause of target inhibition to interact with the cell, therefore underlying mechanism need to be investigated.

Not only the protease inhibition property, rhSLPI itself has been reported to contain anti-oxidant effect that increased the glutathione levels in the lung (Gillissen, et al., 1993, p.825-832). Our results showed that the pretreatment of ARCFs with rhSLPI decreased intracellular ROS generation according to the H_2O_2 challenging. This finding was also similar to the results showed in ARVMs treated with rhSLPI (Prompunt, 2016). However, the possibility of increasing glutathione levels in ARCFs could still inconclusive. Therefore, the level of intracellular antioxidant production after treatment with rhSLPI need to be investigated.

The previous studies documented that myocardial ischemia/reperfusion injury is a potent stimulant of p38 MAPK activation leads to myocardial cell death and myocardial cell injury (See, Kompa, & Krum, 2004, p.149-154; Kumphune, & Chattipakorn, 2012, p.513-524). Our result showed that the treatment of 1 µg/ml of rhSLPI could reduce p38 MAPK activation, hence reduced in cell death when subjected to simulated ischemia/reperfusion. However, protease enzyme activity, ROS and p38 MAPK just only some parts of mechanism in I/R injury, therefore, the other mechanisms e.g. apoptosis pathway, ion channal need further more investigated

One of the interesting issue is the results from this study only showed in an *in vitro* model as determination effect of rhSLPI in ARCFs subjected to sI/R that could protect itself. So, the further investigation should be performed to construct the overexpressing rhSLPI- ARCFs and determine effect of secretome to protect cardiomyocytes, which could provide more information and closely real physiological in the heart.

Conclusion

In summary, this study showed for the first time that the anti-ischemia effect of rhSLPI, which was demonstrated in primary isolated adult rat cardiac fibroblasts. The pretreatment of recombinant human secretory leukocyte protease inhibitor (rhSLPI) exhibits cardioprotective effects, which reduce cell death and cellular ROS production on sI/R injury model. In addition, pretreatment of rhSLPI could reduce cell death by down regulating p38 MAPK.

Acknowledgement

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