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รายงานวิจัยฉบับสมบูรณ์

ชื่อโครงการ ผลของการเพิ่มการแสดงออกและกลไกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสต่อการบาดเจ็บของเซลล์เอนโดธีเลียมในภาวะขาดเลือดจำลอง

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บทสรุปผู้บริหาร

โครงการศึกษาวิจัยเรื่องผลของการเพิ่มการแสดงออกและกลไกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสต่อการบาดเจ็บของเซลล์เอนโดธีเลียมในภาวะขาดเลือด มีวัตถุประสงค์เพื่อค้นหาข้อมูลที่เป็นความรู้พื้นฐานทางวิทยาศาสตร์การแพทย์ โดยการเพิ่มการแสดงออกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสในเซลล์เอนโดธีเลียมจากผลการศึกษาพบว่าสามารถลดการบาดเจ็บของเซลล์เอนโดธีเลียมในภาวะขาดเลือดจำลองได้ และเมื่อนำอาหารเลี้ยงเซลล์ที่ได้จากเอนโดธีเลียมที่มีการแสดงออกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสเพิ่มขึ้นหรือนำเซลล์เอนโดธีเลียมมีการแสดงออกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสเพิ่มขึ้นเลี้ยงร่วมกับเซลล์กล้ามเนื้อหัวใจ พบว่าสามารถลดการบาดเจ็บและการตายของเซลล์กล้ามเนื้อหัวใจอย่างมีนัยสำคัญทางสถิติ จากการศึกษานี้ชี้ให้เห็นว่าเซลล์เอนโดธีเลียมที่มีการแสดงออกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสเพิ่มขึ้นสามารถปกป้องการบาดเจ็บและการตายของเซลล์เอนโดธีเลียมและกล้ามเนื้อหัวใจในภาวะขาดเลือด ข้อมูลที่ได้จากการศึกษาครั้งนี้สามารถนำไปศึกษาต่อยอดทั้งในระดับเนื้อเยื่ออวัยวะและในสัตว์ทดลอง เป้าหมายสุดท้ายของโครงการนี้เพื่อให้เกิดองค์ความรู้ที่จะนำไปสู่การประยุกต์ใช้ในการออกแบบการรักษาทางคลินิกสำหรับผู้ป่วยโรคหลอดเลือดหัวใจที่เสี่ยงต่อการเกิดภาวะกล้ามเนื้อหัวใจตายเฉียบพลันในอนาคต

บทคัดย่อ

การขาดเลือดและการไหลเวียนเลือดกลับในกล้ามเนื้อหัวใจก่อให้เกิดภาวะเครียดออกซิเดชันและเพิ่มการสร้างโปรตีเอสเอนไซม์จากกระบวนการอักเสบ การบาดเจ็บจากภาวะขาดเลือดและการไหลเวียนเลือดกลับนอกจากมีผลต่อเซลล์กล้ามเนื้อหัวใจโดยตรงยังมีผลต่อเซลล์อื่นๆ ที่เป็นองค์ประกอบของเนื้อเยื่อหัวใจโดยเฉพาะอย่างยิ่งเซลล์เยื่อบุหลอดเลือดเอนโดธีเลียมซึ่งนำไปสู่การเพิ่มการบาดเจ็บที่รุนแรง ตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสเป็นเปปไทด์ที่ยับยั้งเฉพาะต่อซีรีนโปรตีเอส จากการศึกษาพบว่ามีบทบาทในการต้านอนุมูลอิสระ ดังนั้นตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสน่าจะมีประสิทธิภาพในการปกป้องหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือดและการไหลเวียนเลือดกลับ วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อประเมินผลการปกป้องการบาดเจ็บจากภาวะขาดเลือดและการไหลเวียนเลือดกลับ โดยการเพิ่มการแสดงออกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสในเซลล์เอนโดธีเลียม รวมทั้งการลดการบาดเจ็บของเซลล์กล้ามเนื้อหัวใจในภาวะขาดเลือดและการไหลเวียนเลือดกลับ การสร้างเซลล์เอนโดธีเลียมที่มีการเพิ่มการแสดงของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสคงที่จากเซลล์สายพันธ์ อีเอเอชวาย 926 โดยศึกษาผลของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสที่ถูกหลั่งจากเซลล์เอนโดธีเลียมต่อการบาดเจ็บของเซลล์กล้ามเนื้อหัวใจสายพันธ์เอช 9 ซี 2 ทั้งในภาวะจำลองการขาดเลือดและการไหลเวียนเลือดกลับในหลอดทดลองและในภาวะพร่องออกซิเจนและการเติมออกซิเจนกลับ

ผลการศึกษาพบว่าการเพิ่มการแสดงออกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสในเซลล์เอนโดธีเลียมสามารถลดการบาดเจ็บและการสร้างอนุมูลอิสระทั้งในภาวะจำลองการขาดเลือดและการไหลเวียนเลือกลับในหลอดทดลองและในภาวะพร่องออกซิเจนและการเติมออกซิเจนกลับ เมื่อนำเซลล์เอนโดธีเลียมที่มีการแสดงออกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสเพิ่มขึ้นเลี้ยงร่วมกับเซลล์กล้ามเนื้อหัวใจในอัตรา 1:1 และ 1:3 พบว่าสามารถลดการบาดเจ็บจากภาวะพร่องออกซิเจนและการเติมออกซิเจนกลับอย่างมีนัยสำคัญ เมื่อนำอาหารเลี้ยงจากเซลล์เอนโดธีเลียมที่มีการแสดงออกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสเพิ่มย้ายมาเพื่อใช้เลี้ยงเซลล์กล้ามเนื้อหัวใจพบว่าสามารถลดการบาดเจ็บของเซลล์กล้ามเนื้อหัวใจจากภาวะพร่องออกซิเจนและการเติมออกซิเจนกลับ โดยผ่านกลไกการลดการหลั่งอนุมูลอิสระ ลดสัดส่วนของโปรอะพอพโทซิสโปรตีนแบกซ์ต่อบีซีแอล 2 และเพิ่มการฟอสโฟรีเลชั่นของเอเคทีไคเนส จากผลการศึกษาสรุปได้ว่าตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสที่ถูกสร้างจากเซลล์เอนโดธีเลียมสามารถปกป้องเซลล์เยื่อบุหลอดเลือดและเซลล์กล้ามเนื้อหัวใจจากภาวะการขาดเลือดและการไหลเวียนเลือดกลับ การเพิ่มการแสดงออกของตัวยับยั้งซีครีโทริลิวโคไซต์โปรตีเอสในเซลล์เยื่อบุหลอดเลือดเอนโดธีเลียมน่าจะเป็นแนวทางใหม่สำหรับการลดการบาดเจ็บของเซลล์ที่เป็นองค์ประกอบหลักของหัวใจจากภาวะการขาดเลือดและการไหลเวียนเลือดกลับ

Abstract

Myocardial ischemia/reperfusion (I/R) induces oxidative stress and over-production of protease enzymes from inflammatory process. I/R injury affects not only cardiomyocytes (CMs) but also surrounding cells, especially vascular endothelial cells (ECs) leading to the aggravation of injury. Secretory leukocyte protease inhibitor (SLPI) is a selective peptide inhibitor for serine proteases. Previous studies showed its role as ROS scavenger. These evidence supported the hypothesis that SLPI could be a potential strategy for cardioprotection on myocardial I/R. So, the aim of this study was to investigate the protective effects of the rhSLPI in ECs, by mean of overexpression, to reduce CMs injury in I/R. The stable endothelial cell line overexpressing human SLPI was established from the human umbilical vein cell line, EA.hy926. The effect of SLPI secreted from ECs on rat cardiac myoblast cells (H9c2) injury was performed in an in vitro simulated I/R (sI/R) or hypoxia/reoxygenation (H/R). The results showed that overexpression of SLPI in ECs could reduce sI/R and H/R induced ECs injury and ROS production. Co-culture of ECs overexpressing SLPI with H9c2 at ratio 1:1 and 1:3 significantly reduced H/R-induced cell injury. Furthermore, transferred media from ECs overexpressing SLPI also significantly reduced H/R-induced H9c2 injury by reducing intracellular ROS production and pro-apoptotic Bax/Bcl-2 ratio, and increasing Akt phosphorylation. In conclusion, SLPI producing from ECs could protect CMs from I/R injury and could possibly be a novel therapeutic strategy for attenuate cardiac cells injury in I/R.

Keywords: Ischemia/reperfusion injury; secretory leukocyte protease inhibitor; protease inhibitor; cardioprotection; endothelial cells; cardiac myocytes

Introduction

Myocardial ischemia/reperfusion (I/R) is a pathological process of cardiomyocytes (CMs) involving in inflammation (1). I/R injury is predominantly caused by oxidative stress, which stimulate infiltration of leukocytes to the injured area. I/R injury could affect not only the CMs, but also surrounding cells, especially endothelial cells (ECs) to cause endothelial dysfunction and lead to ECs injury (2, 3). The latter in turn expand the CMs injury and death (4), and worsen the pathogenesis. Several evidence demonstrated that ECs are more sensitive to I/R injury than CMs (3-5). However, ECs itself could play role in cytoprotection for CMs under a hypoxia/reoxygenation (H/R) condition (6). During I/R, the overproduction of ROS can activate ECs to express cell adhesion molecules (CAMs) and increase vascular permeability (4, 7), which recruit and promote neutrophils trapping to the blood vessel and migrate within ischemic area (4, 5, 7). In addition, infiltrated leukocyte could produce and secrete various protease enzymes, which is known to aggravate the injury of resident cells (2, 8), especially ECs and CMs. Therefore, any strategies to reduce oxidative stress and anti-protease could attenuate the ischemic severity and safe patients' life.

Secretory leukocyte protease inhibitor (SLPI) is protein habouring inhibitory activity selectively for serine proteases, which is known to counteract with excessive inflammatory responses (9). The previous study showed that adding recombinant human SLPI (rhSLPI) in heart preservative solution could reduce murine myocardial injury and infarction, inflammatory cytokine levels, expression of TNF-α, TGF-β, NF-κB, and protease enzyme activity (10). In addition, our previous reports showed that overexpression of human SLPI in rat cardiac myoblast (H9c2) or treatment with recombinant human SLPI (rhSLPI) in both H9c2 and adult rat ventricular myocytes (ARVMs) could reduce cell death, cell injury from an in vitro I/R injury, by attenuating intracellular ROS production during simulated ischemia (11, 12), as well as reducing infarct size (12). Not only cardiomyocytes, SLPI also protect non-cardiomyocyte such as adult rat cardiac fibroblasts cells (ARCFs) and human umbilical vein endothelial cell (HUVECs) from an in vitro I/R injury (13, 14).

Since the previous study demonstrated that ECs could protect CMs from injury (6), the overexpression of any protective mediator, such as rhSLPI, may enhance the cytoprotective effect to CMs during I/R injury. However, the effect of rhSLPI secreted from ECs on cardiomyocyte survival against I/R injury have never been investigated. Therefore, the purpose of this study is to investigate an in vitro effect of overproduction and secretion of rhSLPI from vascular endothelial cells on cardiac cell subjected to ischemia/reperfusion and its protective mechanisms.

Material and methods

Chemicals and reagents

Dulbecco's Modified Eagle Media (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco (Gibco BRL, Life Technologies, Inc., New York, USA). pCMV3-SLPI-GFPSpark tag plasmid was purchased from Sino biology Inc (Sino biology Inc, Beijing, China). This plasmid is 6848 bp vector containing of cDNA of human SLPI (NM_003064.2, the NCBI reference sequence). The 40% (w/v) polyacrylamide gel, polyvinylidenedifluoride (PVDF) membrane and enhanced Chemiluminescence (ECL) were purchased from Merck Millipore (Merck, Darmstadt, Germany). The antibodies recognizing the phosphorylated-ERK1/2, total-ERK1/2, phosphorylated-p38, total-p38, phosphorylated-Akt, total-Akt, Bax, Bcl2, caspase 3 and caspase 8 were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA). All other chemicals were purchased from Sigma (Sigma, St.Louis, MO, USA).

Cell culture

EA.hy926 and H9c2 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) ATCC-CRL2992 and ATCC-CRL1446, respectively. Cells were cultured in Dulbecco's Modified Eagle Media supplement with 10% fetal bovine serum and 5000 units/ml of penicillin/streptomycin and maintained in humidified atmosphere of 95% air and 5% carbon dioxide at 37oC until grown to 70%-80% confluence. For co-culture experiment, EA.hy926 or EA.hy926 overexpressing SLPI was seeded on the upper chamber of 24-transwell permeable plate (NEST, San Diego, CA, USA) and H9c2 was cultured in lower chamber until performing experiments.

Overexpression and clonal selection of EA.hy926 overexpressing rhSLPI

EA.hy926 was seeded into 6-well plate until reached 70-80% confluence overnight prior to transfection. The cells were transfected with the pCMV3-SLPI-GFPSpark tag plasmid by using a Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, US). After transfection process, EA.hy926 was incubated at 37oC in humidified CO2 incubator for 24 h. Then, the medium was removed and replaced by complete media for further 48 h before changing to DMEM containing 100 μg/ml hygromycin B (Invitrogen, Carlsbad, CA, US) for selecting positive transfectants. The cells were continuous selection in drug and expanded from single clonal until the stable overexpression cell line was established. The stable cells were cultured until cells reach to at least three passage before performing in further experiment.

Measurements of SLPI level by ELISA

The human SLPI production was determined by Quantikine® sandwich ELISA kit purchased from R&D Systems (R&D Systems, Inc, Minneapolis, MN, USA). Briefly, 100 μl of assay diluents RD1Q

and culture media were adding on microplate that pre-coat with the SLPI specific monoclonal antibody. Then, the solution was removed and washed with 400 μl of washing buffer three times. For detection, 200 μl of detection antibody conjugated with horseradish peroxidase (HRP) was added into microplate and incubated at room temperature (RT) for 2 h. After incubation, the unbound detection antibody was removed and washed three times. Then, 200 μl of HRP substrates were added to microplate and incubated at RT for 20 min in the dark. Finally, 50 μl of stop solution was added into microplate and the color in the microplate was determined by spectrophotometer at 450 nm. The concentration of rhSLPI was proportion to the color intensity and calculated into quantitative by compare to the standard curve.

Determination of growth curve and cell viability.

EA.hy926 was seeded at seeding densities of 1.5x103 cells into 96 well cell culture plates. The cell viability was determined by MTT assay daily for 7 days. For MTT assay, the cultured media was removed and replaced with 0.5 mg/ml MTT reagent. The reaction was incubated for 2 h at 37oC. After that, MTT reagent was removed and DMSO was added for solubilized the formazan crystal. The formazan soluble was collected and the determined the optical density (OD) by spectrophotometer at λ490 nm using DMSO as a blank. The growth curve was plot between OD and day. The percentage of difference in reduction between control and treatment cell, at various condition, were calculated following formula.

$$Td = (t2 - t1) \times \frac{log(q2)}{log(\frac{q2}{q1})}$$

Where

q1 = Quantity of the cells at start time (1 unit/h)

q2 = Quantity of the cells at end time (1 unit/h)

t1 = Starting time

t2 = Ending time

Determination of cytoskeleton organization

Wild-type EA.hy926 cells or SLPI overexpressing EA.hy926 were cultured in 8-well chamber slide. Then, cells were washed twice with PBS and fixed with 4% formaldehyde for 30 min. Then, the cells were permeabilized with 0.5% Triton-X 100 for 20 min at RT. After that, cells were incubated with TRITC conjugated phalloidin (Sigma, St.Louis, MO, USA) for 40 min in dark moist chamber subsequently

washes twice with PBS. After washing, the cells were stained with DAPI (Sigma, St.Louis, MO, USA) for 20 min in the dark. The cells will be observed under the fluorescent microscope.

Simulated Ischemia/ reperfusion(sI/R) protocol

Simulated ischemia (sI) was performed by method mentioned in previous studies (11, 12). Wild-type or SLPI overexpressing EA.hy926 were incubated with simulate ischemic basic buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM MgCl2, 0.9 mM CaCl2, and 4.0 mM HEPES) containing 20 mM 2-deoxyglucose, 20 mM sodium lactate, and 1 mM sodium dithionite at pH 6.5. EA.hy926 in both groups was subjected to sI for 40 min following by replacing with completed media and then incubated at 37 °C, 5% CO2 for 24 h reperfusion (sI/R). After reperfusion, the cell viability was determined by MTT assay.

Hypoxia/reoxygenation (H/R) protocol.

The H/R protocol was modified from a previous study (16). Briefly, cells were seeded into a 24- well tissue culture plate at a density of 1.5 × 104 cells/well and left overnight. Then, cells were subjected to H/R using overlaying paraffin liquid on the culture media to mimic the hypoxia condition. Cells were subjected to hypoxia for 1 h and reoxygenated by replacing with completed media for 3 h at 37°C. After reoxygenation, cell viability was determined by MTT assay.

Co-culture protocol

For co-culture was performed using a 24-transwell permeable plate (NEST, San Diego, CA, USA) consisting of upper and lower chambers. H9c2 cells at a density of 1.5 × 104 cells/well were seeded in the lower chamber. Wild type (EA-WT) or SLPI overexpressing EA.hy926 cells (EA-SLPI) at 1.5 × 104 cells/well (ratio CMs:ECs as 1:1) or 4.5 × 104 cells/well (ratio CMs:ECs as 1:3) were seeded in the upper chamber. Cells were cultured together for 48 h before being subjected to H/R.

Determination of intracellular ROS production

The method for determine intracellular ROS production was described previously(12). Briefly, cells were cultured with DMEM in 96-well cell culture plates. The culture media was transferred to the new 96-well microtitre plates and incubated in 37oC. Then, the cells were washed once with PBS before incubating with DMEM containing 25μM carboxy-H2DCFDA in a dark room for 30 min at 37 °C. After that, the medium containing carboxy-H2DCFDA was discarded and the culture media, which was restored, was added back to cells again. Then, cells were incubated with 250 μM H2O2 for 30 min at 37 °C or subjected to sI for 40 min or hypoxia 24 h. The intracellular ROS was determined by measuring the fluorescence intensity by EnSpire Multimode Plate Readers (PerkinElmer,

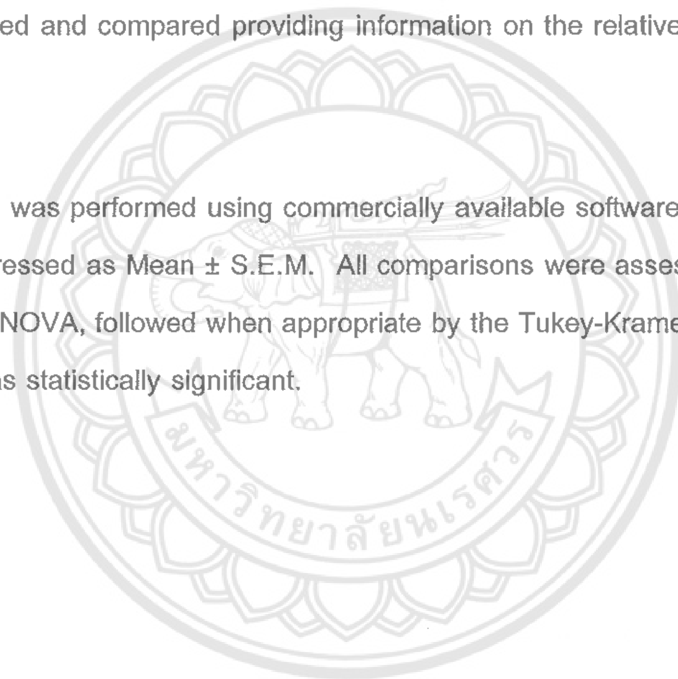
Massachusetts, USA) with the excitation wavelength at λ 498 nm and emission wavelength at λ 522 nm.

Immunoblotting

After H/R, H9c2 that cultured in wild-type or overexpressing SLPI EA.hy926 media were collected protein by adding 2x SDS-sample buffer that containing β-mercaptoethanol as previously described (11). Cells were scraped and transferred to the new pre-cooled microcentrifuge tube. The samples were boiled for 5 min and stored at -20oC untill analysis. Western blots were probed for phosphorylated p38, total-p38, phosphorylated Akt, total-Akt, Bax, Bcl2, caspase 3 and caspase 8, which diluted at 1:1000 in 1% skim milk in TBST buffer at 4oC overnight. The secondary antibodies were either goat anti-mouse or anti-rabbit IgG antibodies conjugated to horseradish peroxidase (HRP) which diluted at 1:5000. The proteins of interest were detected by Chemiluminescence gel documentation. Band densities were quantified and compared providing information on the relative abundance of the protein of interest.

Statistical analysis

The statistical analysis was performed using commercially available software (GraphPad Prism version 5). All data were expressed as Mean ± S.E.M. All comparisons were assessed for significance using an unpaired t-test or ANOVA, followed when appropriate by the Tukey-Kramer test. A p-value less than 0.05 was considered as statistically significant.



Results

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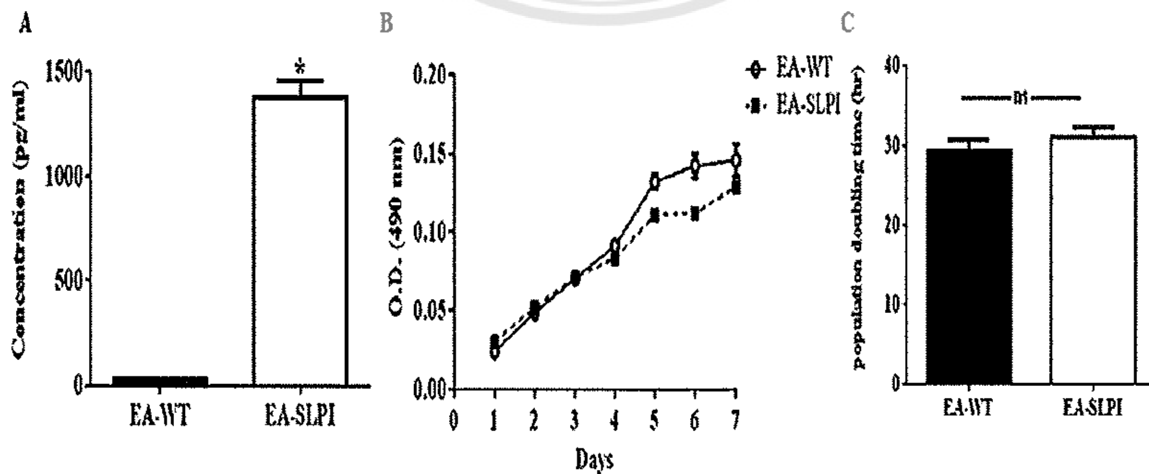
Establishment of stable EA.hy926 cell line overexpressing rhSLPI.

Endothelial cell lines were transfected with pCMV3-SLPI-GFPSpark tag plasmid. There were 4 clones including E3, F5, G3 and G5 that successful selected by limiting dilution in the completed medium containing of 100 µg/ml of hygromycin B. After cells reach to appropriate amount (>80% confluence), cells were determined rhSLPI production by ELISA compare to wild-type. The results showed that G3 are the clone that secreted highest rhSLPI when compare to other clone (data not shown) and was use in further experiments. Moreover, the expression of rhSLPI in stable cells that receive rhSLPI plasmid was significantly increased in the culture media when compare to wild-type (1378 ± 77.80 pg/ml and less than 25 pg/ml, $p < 0.05$) (Figure 1A).

After establishment of SLPI overexpressing EA.hy926 cells, the biological characteristics such as growth curve, population doubling time (PDT), and cell morphology, were measured and compared to the wild type EA.hy926 cells. The proliferation of the wild type EA.hy926 cells, and SLPI overexpressing EA.hy926 cells, were not different when determined by growth curve (Figure 1B). The results also showed that the PDT of those cell lines was not significantly different (Figure 1C). Moreover, the cell morphology of wild type and SLPI overexpressing EA.hy926 were not different in terms of size and shape. Determination of cytoskeleton organization by phalloidin-TRITC staining showed the minor different in SLPI overexpressing EA.hy926 cells by increasing of actin filament dense in the margin of the cells more than the wild type (Figure 1D).



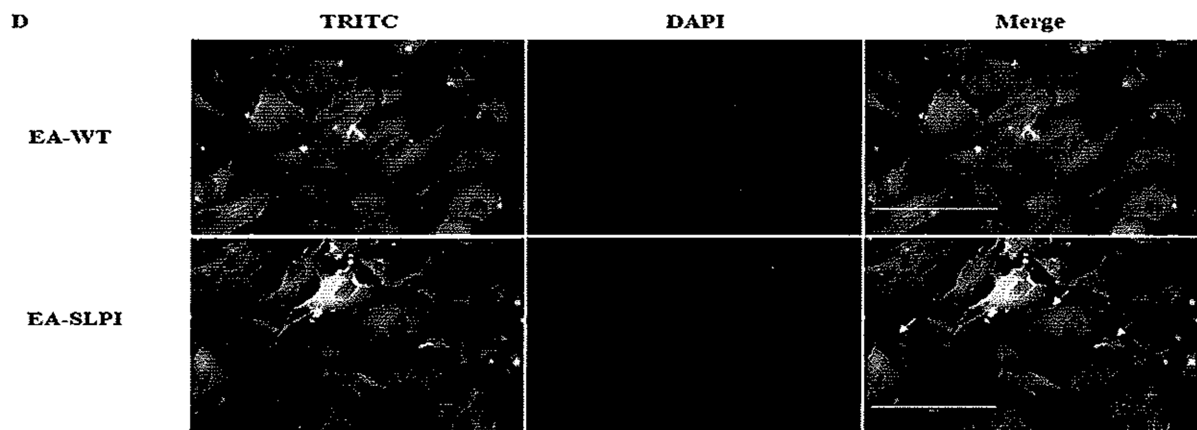


Figure 1. Biological characterization of overexpressing SLPI EA.hy926 cells. EA.hy926 cells were transfected with with pCMV3-SLPI-GFPSpark tag plasmid by using lipofectamine® 2000 and the level of SLPI production, growth curve, PDT and morphology were performed. (A) the level of SLPI production in culture media were determined by using a human SLPI ELISA kit; (B) wild type and overexpressing SLPI EA.hy926 cells were determined by MTT assay every days for 7 days; (C) wild type and overexpressing SLPI EA.hy926 cells were counted on days 3, 5 and 7 to determine the growth curve; (D) the EA.hy926 cells morphology were determined by staining with TRITC-conjugated phalloidin and DAPI and visualized under fluorescence microscopy. Each bar graph represents the means ± S.E.M for each of the three experiments. $*p < 0.05$ vs wild type group (t-test).

Overexpression of SLPI reduced vascular endothelial cells injury against in vitro simulated ischemia/reperfusion (sI/R)

After stable endothelial cell line was established, the protective effect of rhSLPI during simulated ischemia/reperfusion (I/R) on endothelial cell was determined. Before simulated ischemia/reperfusion (sI/R) experiment was performed, the optimized time of sI/R that could cause 50 % of cell death were optimized. EA.hy926 cells were exposed to sI buffer for several periods and followed by reperfusion for 24 h. The results showed that simulated ischemia reduced the cell viability in time-dependent manner (Figure 2A). The sI/R at 40 min were reduced the cell viability by ~50% (57.09 ± 0.78 %, $p < 0.05$). So, this time point was used in sI/R protocol for further experiment. Then, both of wild type EA.hy926 and SLPI overexpressing EA.hy926 cells were subjected to 40 min of sI buffer and followed by 24 h of reperfusion. Then, the relative cell viability was determined by MTT assay. The results

showed that the relative percentage of cell viability of the SLPI overexpressing EA.hy926 cells (81.75 ± 1.42 %, p< 0.05) were significantly higher than that wild type EA.hy926 cells (60.27 ± 2.52%, p< 0.05) (Figure 2B).

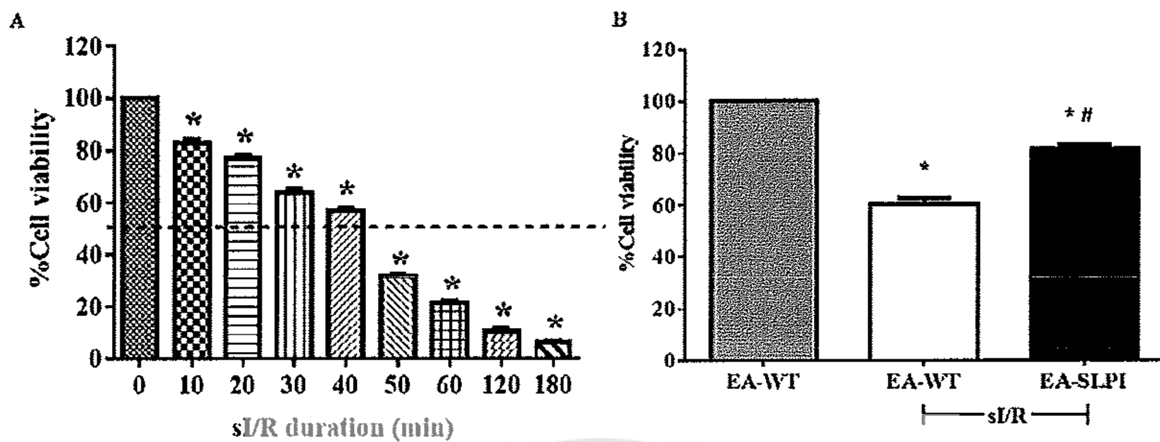


Figure 2. Determination of overexpressing SLPI EA.hy926 cells in ischemia/reperfusion (I/R). (A) EA.hy926 cells were subjected to 10-180 min simulated ischemia (sI) followed by 24 h of reperfusion; (B) wild type and overexpressing SLPI EA.hy926 cells were exposed to 40 min of sI followed by 24 h reperfusion and determined the cell viability by MTT. Each bar graph represents the means ± S.E.M for each of the three experiments. *p < 0.05 vs not sI/R treated group (ANOVA), # p < 0.05 vs among sI/R treated group (ANOVA).

Overexpression of SLPI reduced hypoxia/reoxygenation induced vascular endothelial cellular injury

Because rhSLPI was secreted from the cells to media and we need to determine the rhSLPI effects that protect themselves and other side-cells when myocardial I/R occurred. The sI/R model has to remove the media and replace with sI buffer leading to loss of rhSLPI during sI/R injury. Therefore, the hypoxia/reoxygenation (H/R) model was used as the model in this study that mimics the process of sI/R when oxygen did not supply to the tissue. Before H/R was performed, the optimized time of H/R that could cause 50 % of cell death were optimized by overlay the liquid parafilm cover the culture media surface (hypoxia) in several periods followed by reoxygenation for 3 h. The results demonstrated that hypoxia also reduced the cell viability in a time-dependent manner. The hypoxia at 1 h were significantly reduced the cell viability to ~50% (52.99 ± 2.47 %, p< 0.05) when compared to control (Figure 3A). Thus, H/R at 1 h/3 h duration was used in H/R protocol in further experiment.

After H/R optimization, both of cell groups were exposed to H/R and cell viability were determined by MTT assays, the results showed that the relative percentage of cell viability of the SLPI overexpressing EA.hy926 cells (83.57 ± 1.78 %, $p < 0.05$) was significantly higher than that of wild type EA.hy926 cells (63.07 ± 1.93%, $p < 0.05$) (Figure 3B).

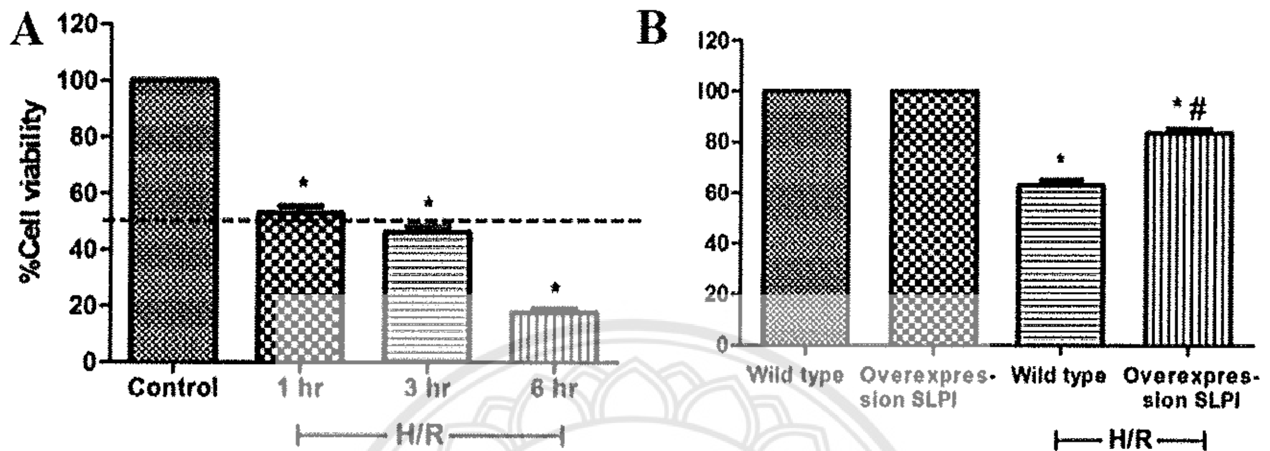


Figure 3. Determination of overexpressing SLPI EA.hy926 cells in hypoxia/reoxygenation (H/R). (A) EA.hy926 cells were subjected to 1, 3 and 6 h hypoxia followed by 3 h of reoxygenation; (B) wild type and overexpressing SLPI EA.hy926 cells were exposed to 1 h of hypoxia followed by 3 h reoxygenation and determined the cell viability by MTT. Each bar graph represents the means ± S. E. M for each of the three experiments. *$p < 0.05$ vs not H/R treated group (ANOVA), # $p < 0.05$ vs among H/R treated group (ANOVA).

Overexpression of SLPI reduced sI/R induced intracellular ROS production in vascular endothelial cells

Previous studies reported that SLPI act as ROS scavenger by reducing intracellular ROS production in many cells during sI/R (11-14). To proof the effect of SLPI secreted from SLPI overexpressing EA.hy926 cells could reduce intracellular ROS production, the in vitro intracellular ROS production was performed under sI/R, H/R and H2O2 challenging were investigated. The results showed that sI/R significantly increased the relative intracellular ROS level (1.91 ± 0.12, $p < 0.05$) when compared to overexpression SLPI group (1.55 ± 0.06, $p < 0.05$) (Figure 4A). Moreover, the results from H/R showed that H/R significantly increased the relative intracellular ROS level in control cells (1.55 ±

0.06, p< 0.05) when compared to SLPI overexpressing EA.hy926 cells (1.37 ± 0.03, p-value< 0.05) (Figure 4B). Similar to sI/R and H/R result, H2O2 challenge in both group of EA.hy926 significantly increased the relative intracellular ROS level (32.85 ± 2.28, p-value< 0.05) when compared to SLPI overexpressing EA.hy926 cells (17.01 ± 0.56, p-value<0.05) (Figure 4C).

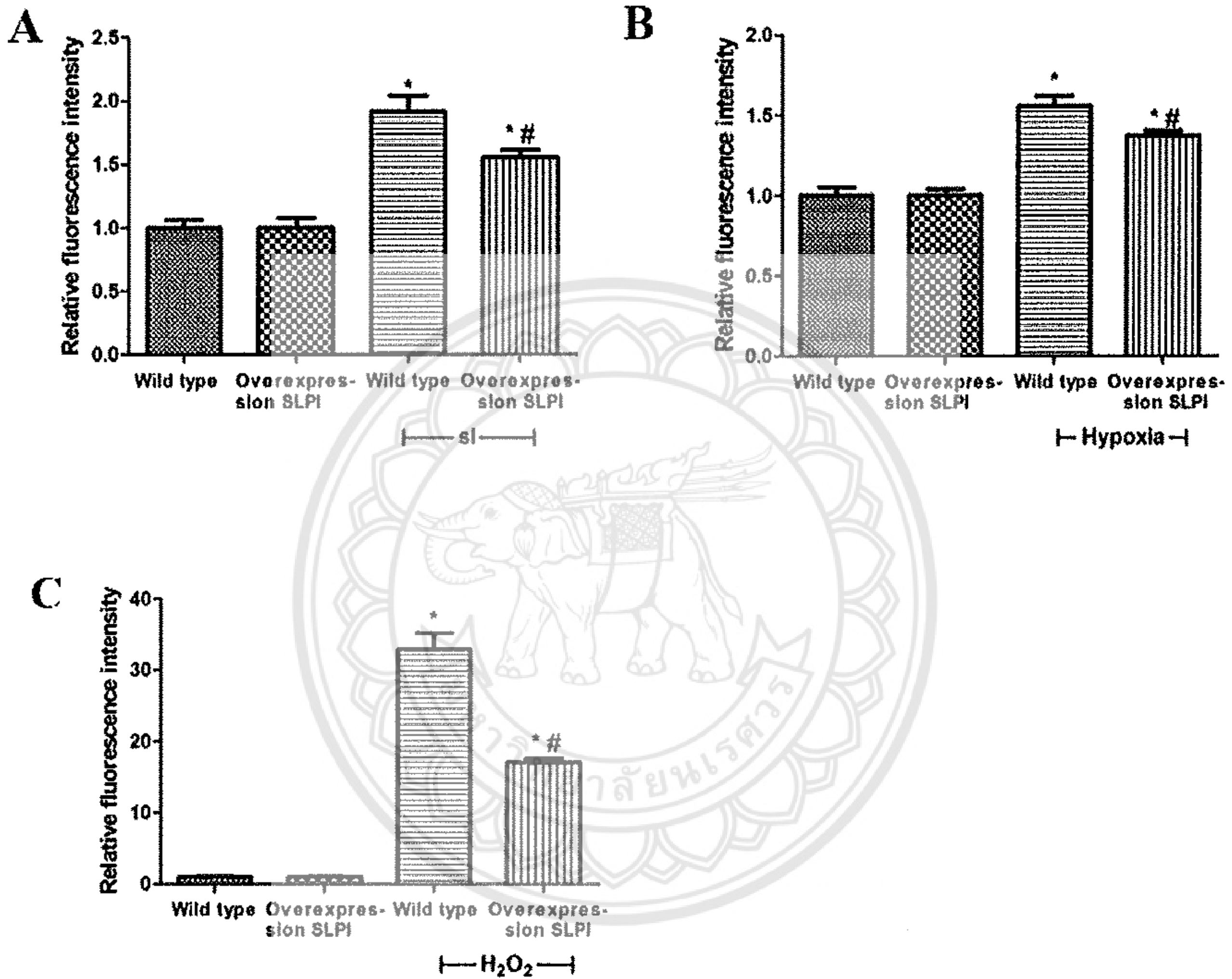


Figure 4. Determination of intracellular ROS level in overexpressing SLPI EA.hy926 cells. Wild type and overexpressing SLPI EA.hy926 cells were exposed to H2DCFDA followed by (A) sI/R or (B) H/R or (C) H2O2 challenge. Then, the intracellular ROS level was determined by spectrophotometric. Each bar graph represents the means ± S.E.M for each of the three experiments. *p < 0.05 vs not treated group (ANOVA), # p < 0.05 vs among treated group (ANOVA).

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rhSLPI secreted from SLPI overexpressing endothelial cells protected cardiomyocytes from hypoxia/reoxygenation induced cell injury

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To investigate the cardio-vasculoprotective effect of SLPI, the wild type cells and SLPI overexpressing EA.hy926 cells were co-culture with rat cardiac myoblast H9c2 cells with the ratio of initial cell density at 1:1 and 1:3 before subjected to 1 h of hypoxia followed by 3 h of reoxygenation. The results showed that the percentage of cell viability of H9c2 co-cultured with wild type EA.hy926 or SLPI overexpressing EA.hy926 cells were significantly higher than H9c2 alone (52.82 ± 0.96%, $p < 0.05$) (Figure 5A). Interestingly, H9c2 that co-culture with SLPI overexpressing EA.hy926 cells in both ratio (1:1 = 78.46 ± 2.90%, 1:3 = 82.17 ± 1.63%, $p < 0.05$) were significantly increased in cell viability when compared to H9c2 that co-culture with wild type EA.hy926 (1:1 = 67.95 ± 1.27%, 1:3 = 65.47 ± 1.22%, $p < 0.05$).

To confirmed that secreted SLPI from ECs could provide cytoprotective factors to reduce CMs injury, the culture medium from wild type and SLPI overexpressing EA.hy926, with initial seeding density similar the density in co-culture experiments of 1:1 and 1:3 ratio, were transferred to culture H9c2 cells before exposing to H/R. The results showed that H9c2 cells that culture in transferred media from SLPI overexpressing EA.hy926 cells in both original density ratio (1:1 = 75.42 ± 4.74%, 1:3 = 82.43 ± 4.06%, $p < 0.05$) were significantly increased in cell viability when compared to H9c2 cultured in transferred media from wild type EA.hy926 (1:1 = 60.17 ± 4.09%, 1:3 = 61.98 ± 4.34%, $p < 0.05$) (Figure 5B).

Moreover, to elucidate the effect of rhSLPI from transferred media on intracellular ROS production, the intracellular ROS level was determined. In H9c2 cell cultured with transferred media from wild type and SLPI overexpressing EA.hy926 cells at original density ratio 1:1 and 1:3 to H9c2, the cells were exposed to H2DCFDA followed by H/R. The results showed that H9c2 cells that cultured in transferred media from SLPI overexpressing EA.hy926 cells in both original density ratio (1:1 = 0.80 ± 0.05, 1:3 = 0.82 ± 0.05, $p < 0.05$) were significantly decreased in relative intracellular ROS level when compared to H9c2 that rhSLPI secreted from endothelial cells protected cardiomyocytes cell from hypoxia/reoxygenation injury via Akt, and p38MAPK signaling pathway.

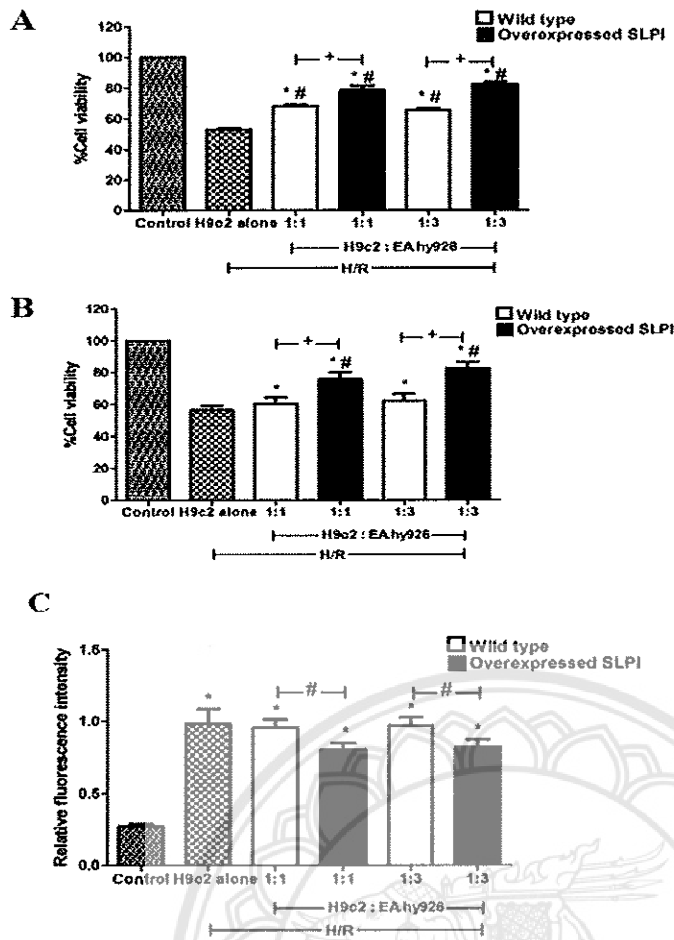

Figure 5. The effect of SLPI secreted from endothelial cells line protected cardiomyocyte cell line from hypoxia/reoxygenation. (A) wild type and overexpressing SLPI EA.hy926 cells were co-culture with H9c2 cell line at ratio 1:1 and 1:3 then subjected to H/R and determined the cell viability by MTT; (B) the media that culture wild type and overexpressing SLPI EA.hy926 cells were transferred to culture H9c2 cells at ratio 1:1 and 1:3 then exposed to H/R and determined the cell viability by MTT. After transfer media from wild type and overexpressing SLPI EA.hy926 cells at ration 1:1 and 1:3 to H9c2 cells, (C) the cells were exposed to H2DCFDA followed by H/R and determined the intracellular ROS by spectrophotometric. Each bar graph represents the means ± S.E.M for each of the three experiments. *$p < 0.05$ control group (ANOVA), # $p < 0.05$ vs H9c2 alone group (ANOVA), +$p < 0.05$ vs among culture ratio group (t-test).

To determine the effect of rhSLPI in cellular signaling, the media that culture wild type and SLPI overexpressing EA.hy926 cells at initial seeding density of 1:3 were transferred to culture H9c2 cells before exposure to H/R. The results showed that H9c2 cells that culture in transferred media from SLPI

overexpressing EA.hy926 cells significantly increase the phosphorylation of p38MAPK when compare to the control and H9c2 that culture in transferred media from EA.hy926 wild type but not significantly different when compare to H9c2 alone (Figure 6A). Moreover, H9c2 cells that culture in transferred media from SLPI overexpressing EA.hy926 cells significantly increase the phosphorylation of Akt when compare to the control, H9c2 that culture in transferred media from EA.hy926 wild type and H9c2 alone (Figure 6B).

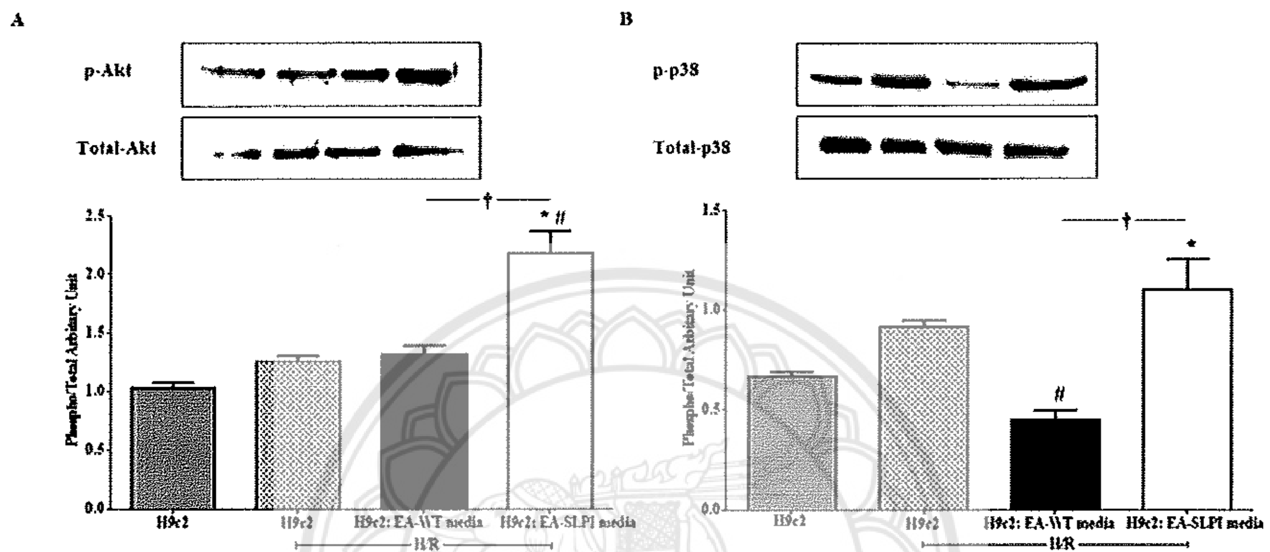

Figure 6. Determination the effect of rhSLPI secreted from endothelial cells on cardiomyocytes cell line subjected to H/R in cellular signaling response. The media that culture wild type (EA-WT) or overexpressing SLPI (EA-SLPI) EA.hy926 cells were transferred to culture H9c2 cells then exposed to H/R and the activation of Akt (A) and p-38 MAPK (B) were determined by Western blot analysis. Each bar graph represents phosphorylation ratio (phospho/total) of Akt and p38 MAPK. *p < 0.05 H9c2 that not subjected to H/R group (ANOVA), # p < 0.05 vs H9c2 that subjected to H/R group (ANOVA), †p < 0.05 vs H9c2:EA-WT media group (ANOVA, n =3).

rhSLPI secreted from endothelial cells decreased cardiomyocyte cell death from hypoxia/reoxygenation injury through Bax and Bcl apoptosis pathway. Previous results indicated that secreted SLPI from EA.hy926 can reduce H/R induced H9c2 cell death. To elucidate the protective effect of rhSLPI in cell death, apoptosis signaling were performed. The result showed that H/R increased the Bax/Bcl-2 ratio in H9c2 alone when compared to the control. In contrast, H9c2 that culture in transferred media from wild type EA.hy926 and SLPI overexpressing EA.hy926 cells significantly decreased relative Bax/Bcl-2

ratio when compared to H9c2 alone. Furthermore, H/R also increased caspase-3 expression in H9c2 alone when compared to the control group (Figure 7A). However, only H9c2 that culture in transferred media from wild type EA.hy926 decreased caspase-3 expression when compared to H9c2 alone (Figure 7B).

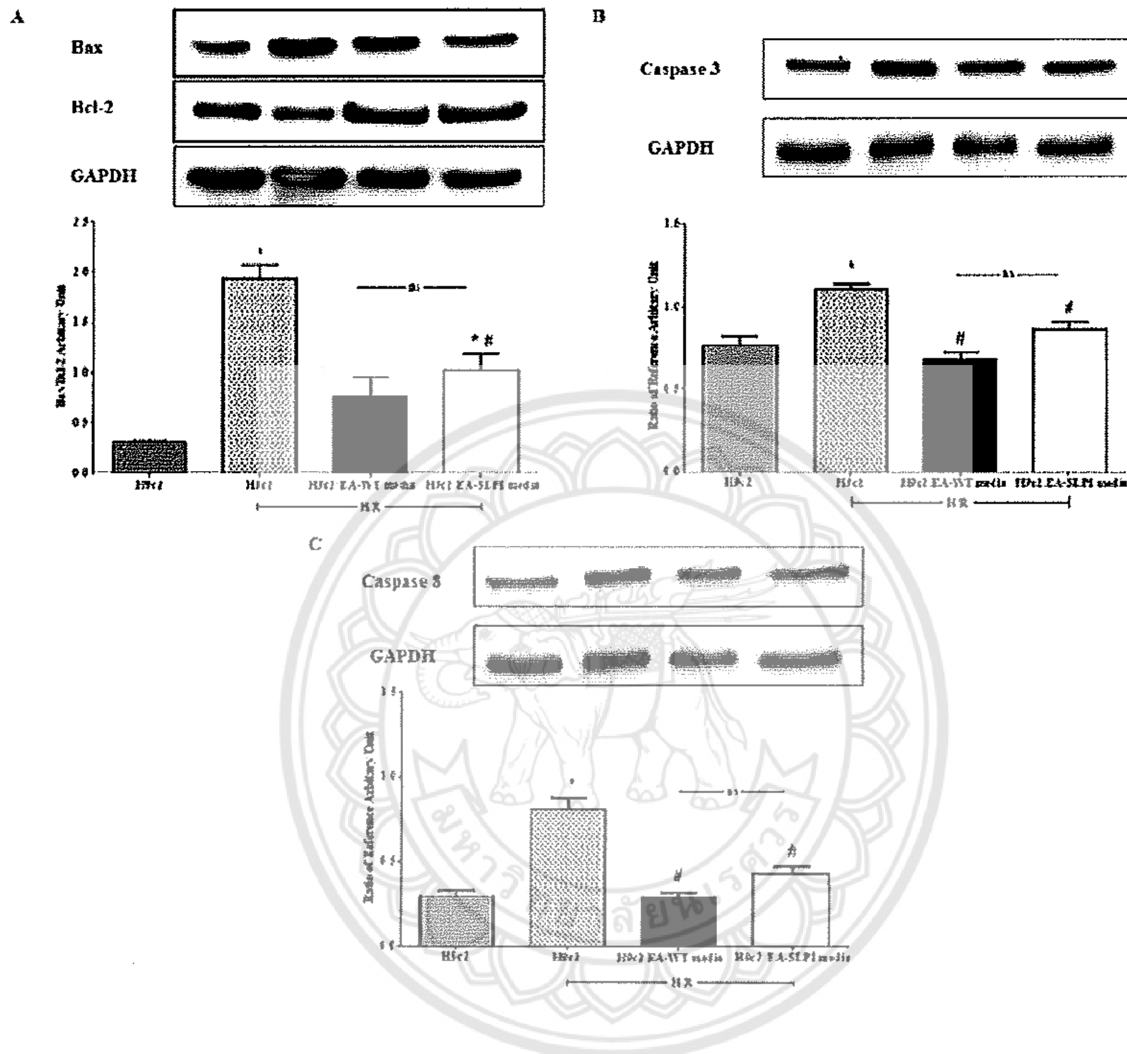

Figure 7. Determination the effect of rhSLPI secreted from endothelial cells on cardiomyocytes cell line subjected to H/R in apoptosis pathway. The media that culture wild type (EA-WT) or overexpressing SLPI (EA-SLPI) EA.hy926 cells were transferred to culture H9c2 cells then exposed to H/R and the apoptotic protein including Bax/Bcl-2 (A) caspase 3 (B) and caspase 8 (C) were determined by Western blot analysis. Each bar graph represents relative ratio of reference protein (GAPDH). *$p < 0.05$ H9c2 that not subjected to H/R group (ANOVA), # $p < 0.05$ vs H9c2 that subjected to H/R group (ANOVA, n =3).

Discussion

The present study focus on the potential therapeutic effect of recombinant human secretory leukocyte protease inhibitor (rhSLPI) that secreted from vascular endothelial cell to protect cardiac cells during ischemia/reperfusion (I/R) injury and also in hypoxia/reoxygenation (H/R) injury. The major findings of this study were the overexpression of human SLPI cDNA in vascular endothelial cells could reduce myocardial I/R and also H/R-induced ECs death via attenuation of intracellular ROS production. Moreover, rhSLPI secreted from ECs could protected H/R-induced CMs death and also decrease intracellular ROS production by activation of Akt phosphorylation and decreased pro-apoptotic Bax/Bcl-2 protein.

Cardiovascular system is composing of heart and vascular. In the heart, there are consisting of various cell types such as cardiomyocytes (CMs), vascular endothelial cells (ECs), vascular smooth muscle cells (SMCs), cardiac fibroblast and pericytes (16). Each of these cells plays an important role in both physiological and pathological condition (16). Previous studies reported that ECs were the major population which found in the mouse heart and can quantitate into 45% of non-myocyte population (17). In addition, ECs are more tolerance than CMs to ischemia but there are sensitive to I/R injury (5, 18). Moreover, I/R injury in rat heart in different time showed that ECs in small coronary vessel were the early cell population undergo to apoptosis followed by a radial spread of CMs apoptosis (19). This phenomenon implied that I/R could induce ECs to release some paracrine mediators to cause CMs death (19). However, co-culture of ECs and CMs subjected to H/R showed the ECs-derived protection of CMs by increase nitric oxide production and also decrease lactate dehydrogenase (LDH) activity when compare to CMs alone (6). Thus, cytoprotection of ECs or manipulation of ECs to secret some cytoprotective factors could be therapeutic strategies to reduce the severity of myocardial I/R injury.

Several studies showed the cytoprotective effect of rhSLPI in myocardial I/R injury including cardiomyocyte, cardiac fibroblast and vascular endothelial cells (10-14). The firstly reported in 2008 by Schneeberger S. et al. indicated rhSLPI in cold-preservative buffer during cardiac transplantation in mice could benefit to the transplanted heart by improving cardiac score, decreased protease enzyme and pro-inflammatory cytokines expression in transplanted mice (10) leading to an attractive effect of rhSLPI in I/R injury. Our previous reports also demonstrated the beneficial effect of overexpression of SLPI in cardiac cell line could reduce I/R induce cell death, cell injury, and intracellular ROS production (11). In addition, an in vitro rhSLPI treatment in adult rat cardiac fibroblasts (ARCFs) and human

umbilical vein ECs (HUVECs) could also protect both type of cells from I/R injury (13, 14). However, the effect of overexpression of SLPI in ECs to protect itself as well as the role of secreted SLPI from ECs as a cytoprotective factors to reduce the I/R induced CMs injury has never been investigated.

Due to less expression of SLPI in vascular endothelial cell line EA.hy926, the results indicated that introduction of rhSLPI cDNA could successfully overexpress SLPI in EA.hy926 cell line. The EA.hy926 cell line is hybrid cells derived by fusing HUVECs with the permanent human cell line A549 (20). Overexpression rhSLPI ECs have morphology similar to primary endothelial cell or cobblestone shape (20, 21). The genetic manipulation by overexpression of gene could effect on cellular biological properties of the cells. In this study, the cellular biology of EA.hy926 cells overexpressing SLPI was not significantly changed in term of growth rate, population doubling time, size, and shape. But the cytoskeleton organization showed a slight change in organization when observed by phalloidin staining (Figure 1D). The actin exhibited a circumferential shape and was mostly surrounding near the cell margin. This change could possibly increase the adhesion, proliferation, as well as migration (22).

Overexpression of rhSLPI in vascular endothelial cell induce cell death and attenuated intracellular ROS production. This finding was similar to the previous finding of treatment of rhSLPI in HUVECs (13). However, in our previous studies showed the most effective concentration of rhSLPI in pretreatment procedure was 1000 ng/ml (13, 14), but the culture condition in this study could give the level secreted rhSLPI only in nanagram per milliliter (Figure 1A).

Since the stable SLPI overexpressing cells secreted rhSLPI into the media, therefore co-culture between ECs overexpressing SLPI and CMs, as well as determining the effect of transferred culture media from ECs overexpressing SLPI to CMs were performed. These experiments aimed to prove the hypothesis that secreted SLPI from ECs could provide cytoprotective factors to reduce H/R induced CMs injury. It has been known that in physiological heart, the ratio of EC to CM is approximately 3:1 (23). Leucker TM et al. found that co-culture ECs to CMs at ratio 3:1 significantly decreased H/R induced-CMs injury (6). This was similar to our findings that co-culture of CMs with control ECs, at density ratio 1:1 and 1:3, could significantly reduce H/R induced-CMs death when compare to the condition without ECs (Figure 5A). Interestingly, co-culture of CMs with ECs overexpressing SLPI at both ratios could enhance the protective effect against H/R induce CMs injury. From this results, indicating that secreted rhSLPI have synergized effect with other cytoprotective factors secreted from ECs to reduce H/R induced ECs injury.

Furthermore, the cultured medium collected ECs overexpressing SLPI, at the initial cell density ration of 1:1 and 1:3 conditions, was also protect CMs from H/R injury. Our finding showed that rhSLPI secreted from ECs could significantly enhance the protective effect to reduce CMs injury. This suggested that manipulation of ECs to produce and secrete SLPI could be a possible alternative therapeutic strategy to reduce the severity of myocardial I/R injury.

In this study, we focused on the roles of rhSLPI that secreted from ECs to protect themselves and other neighboring cells, especially CMs, subjected to H/R injury. Because of rhSLPI is a secreted from the cells and accumulated in the media (Figure 1A), using sI/R in the experiment might not be appropriate because the protocol need to remove the culture media and replace with simulated ischemic (sI) buffer. This mean that rhSLPI secreted in culture medium were wash out and the effect of rhSLPI during sI/R could be misinterpreted. So, an alternative study model using hypoxia/reozygenation (H/R) model to mimic the myocardial I/R injury was performed. H/R model is a well-known and accepted by several studies to mimic in vitro cellular injury in ischemia/reperfusion (24, 25) according to the ability of H/R to induce cellular apoptosis. However, H/R might not represent the real physiological event in myocardial I/R injury. Therefore, an in vivo study of myocardial I/R injury such as by left anterior descending (LAD) coronary artery ligation need to be investigated.

Myocardial I/R injury induced cell death via several signaling pathway, predominantly on p38 MAPK (26, 27), Bax, Bcl-2, and caspase cascades which defined as mediator of cellular apoptosis (28). Activation of p38 MAPK is well known to cause myocardial cell death and injury (28, 29). However, several evidences suggested that activation of p38 MAPK could provide cardioprotection may according to dominate ischemic preconditioning (IPC) (30, 31). Our results found that transferred media of ECs overexpressing SLPI could activate p38 MAPK in CMs (Figure 6B). Furthermore, rhSLPI secreted from ECs could protect CMs from apoptosis by reducing pro-apoptotic protein Bax/Bcl-2 ratio and caspase-3 (Figure 7), and activation of pro-survival kinase Akt. These findings were similar to our previous reports demonstrated in sI/R stress pre-treatment of rhSLPI in various cardiac cell types or overexpression SLPI in CMs significantly activated Akt signaling (11, 13). However, determination of apoptotic cell death was not performed in this experiments and was considered as the limitation of the study.

Conclusion

This is the first study to demonstrate the manipulation of ECs to secret rhSLPI could provide cardio-vasculoprotection against I/R and H/R injury. The protective effects of secreted rhSLPI could be due to the protection of endothelial cells itself injury and also CMs by attenuation of intracellular ROS production, pro-apoptotic Bax/Bcl-2 and caspase-3, and activation of pro-survival Akt phosphorylation. This study suggested the SLPI could be considered as a novel alternative therapeutic strategy to reduce the severity of myocardial I/R injury.

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