AN *IN VIVO* EFFECT OF RECOMBINANT HUMAN SECRETORY LEUKOCYTE PROTEASE INHIBITOR IN MYOCARDIAL ISCHAEMIA/REPERFUSION INJURY

PODSAWEE MONGKOLPATHUMRAT

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in Biomedical Sciences
2022

AN *IN VIVO* EFFECT OF RECOMBINANT HUMAN SECRETORY LEUKOCYTE PROTEASE INHIBITOR IN MYOCARDIAL ISCHAEMIA/REPERFUSION INJURY

PODSAWEE MONGKOLPATHUMRAT

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in Biomedical Sciences
2022

Thesis entitled "An *in vivo* effect of recombinant human secretory leukocyte protease inhibitor in myocardial ischaemia/reperfusion injury"

By Podsawee Mongkolpathumrat

has been approved by the Graduate School as partial fulfillment of the requirements

for the Doctor of Philosophy in Biomedical Sciences of Naresuan University

Oral Defense Committee

..... Chair
(Stéphanie Barrère-Lemaire, Ph.D.)

..... Advisor
(Associate Professor Sarawut Kumphune, Ph.D.)

..... Co Advisor
(Associate Professor Anusak Kijtawornrat, D.V.M., Ph.D.)

..... Co Advisor
(Assistant Professor Nitirut Nernpermpisooth, Ph.D.)

..... Internal Examiner
(Panyupa Pankhong, Ph.D.)

Approved

.....
(Associate Professor Krongkarn Chootip, Ph.D.)
Dean of the Graduate School

Title	AN *IN VIVO* EFFECT OF RECOMBINANT HUMAN SECRETORY LEUKOCYTE PROTEASE INHIBITOR IN MYOCARDIAL ISCHAEMIA/REPERFUSION INJURY
Author	Podsawee Mongkolpathumrat
Advisor	Associate Professor Sarawut Kumphune, Ph.D.
Co-Advisor	Associate Professor Anusak Kijtawornrat, D.V.M., Ph.D. Assistant Professor Nitirut Nernpermpisooth, Ph.D.
Academic Paper	Ph.D. Dissertation in Biomedical Sciences, Naresuan University, 2022
Keywords	Myocardial infarction, Ischaemia/reperfusion, Secretory leukocyte protease inhibitor (SLPI), Post-ischaemic treatment, Cardioprotection, Anti-protease deficient

ABSTRACT

Proteolysis by the protease enzyme aggravates myocardial ischaemia/reperfusion (I/R) injury by expanding the injured area and inducing inflammation. These lead to impaired cardiac function and cardiac structure, which contribute to cardiac remodelling and heart failure. Inhibition of proteases seems to provide therapeutic benefit. Our previous study demonstrated that giving recombinant human secretory leukocyte protease inhibitor (rhSLPI) by means of overexpression of the rhSLPI gene or treatment with recombinant protein of human SLPI provided a cytoprotective effect against I/R injury. Unfortunately, the information mostly came from an *in vitro* and an *ex vivo* experiment with pre-ischaemic treatment. Nevertheless, the roles and consequences of rhSLPI in the post-ischemic period, especially in an *in vivo* model, as well as post-ischaemic cardiac remodelling and hypertrophy have not been intensively investigated. In this study, recombinant human (rh)SLPI was administered intravenously during left anterior descending (LAD) coronary artery ligation and the onset of reperfusion. The results showed that post-ischaemic treatment with rhSLPI could significantly reduce infarct size, lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) activity, inflammatory cytokines, and protein carbonyl levels, as well as improve cardiac functions. The

intracellular mechanistic explanation for the cardioprotection is due to inhibition of p38 MAPK phosphorylation, downregulation of Bax, caspase-3 and 8 protein levels, and activation of Akt and ERK1/2 phosphorylation. In summary, this is the first report showing the cardioprotective effects against myocardial I/R injury of post-ischaemic treatments with rhSLPI *in vivo*.

Although the cardioprotective effect of SLPI is proven, it remains unclear whether the cardioprotective effect of SLPI seen in our previous work is due to inhibition of protease enzymes. Therefore, an anti-protease deficient mutant SLPI has been developed to investigate if the cardioprotective effect is dependent upon its anti-protease activity. In this part, recombinant protein of anti-protease deficient mutant SLPI (L72K, M73G, L74G), or mt-SLPI, was intravenously administered to rats after they were subjected to LAD ligation. The results showed that mt-SLPI could significantly reduce cardiac cell death, and intracellular reactive oxygen species (ROS) production against an *in vitro* simulated I/R injury. Furthermore, post-ischaemic treatment of mt-SLPI could significantly reduce infarct size and cardiac biomarkers such as lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) activity, improve cardiac functions, attenuate I/R induced-p38 MAPK phosphorylation, and reduce apoptotic regulatory protein levels, including Bax, cleaved-Caspase-3 and total Capase-8, in rats subjected to an *in vivo* I/R injury. Additionally, the beneficial effect of mt-SLPI was not significantly different from wild-type (wt-SLPI). Thus, anti-protease deficient mutant SLPI could also provide a cardioprotective effect, which could be more clinically beneficial in terms of providing cardioprotection without interfering with basal serine protease activity.

In summary, post-ischemic treatment of recombinant human SLPI could be used as a future novel therapeutic alternative for myocardial I/R injury.

ACKNOWLEDGEMENTS

First of all, I would like to thank and offer my respect to supervisor Associate Professor Dr. Saruwut Kumphune, who has supported me throughout the length of the study, with his attention, his energy, his patient and his kindness give me helpful advice and technique. I also would like to give special thanks to all thesis committee members, and deeply grateful for their helpful comments.

I wish to acknowledge my colleagues and all staffs at the faculty of Allied Health Sciences, Naresuan University and all staffs of Biomedical Engineering Institute, Chaing Mai University for their encouragement and support. I would like to thank Professor Dr. Stephanie Barrere-Lemaire to become chair in this thesis examination. I would like to thank Dr. Panyupa Pankhong to become committee in thesis examination. I would like to thankful Associate Professor Dr. Anusak Kijtawornrat, who has grateful given me advices in the animal surgery and experimental technique. The friendship and encouragement given to me by my friends and all graduate students in “SK LAB”.

I would also like to show my gratitude to all staffs at the Chulalongkorn University Laboratory Animal Center (CULAC), Chulalongkorn University to provide technical guidance and support throughout the experiments in laboratory animals. In addition, I would like to thank Royal Golden Jubile PhD scholarship for graduate research to support my study and additional grant from French Embrassy contribution to the Royal Golden Jubilee for support my additional experiments in Institute Genomics Functional (IGF), Montpellier, France.

Finally, I want to give specially thank and respect to my family, for their love, understanding and support. I dedicate the honor of this work to them.

Podsawee Mongkolpathumrat

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Introduction

Ischaemic Heart Disease is predicted to be the major cause of death among the population around the world in 2030 (1). This phenomenon seems to happen everywhere in the world, not only in developed countries. According to epidemiological data from Thailand, ischaemic heart disease has become one of the leading causes of death in the Thai population (1). The survivors of myocardial infarction can subsequently suffer from heart failure. Therefore, any strategies that prevent or lower the severity of myocardial ischaemia/reperfusion injury can save patients' lives and improve their quality of life.

Ischemia causes cardiac cells to be injured due to a lack of oxygen and nutrients from the blood, as well as the activation of molecular cascades. Reperfusion therapy is the only intervention that allows the blood to perfuse back to the infarct area, such as with angioplasty, coronary bypass surgery, reducing cellular injury and death. However, reperfusion itself could also cause further injury. Altogether, the injury is known as an ischemic/reperfusion (I/R) injury (2). During myocardial ischaemia and reperfusion, various biochemical processes occur, including post-ischaemic inflammation, the production of oxygen radicals, polymorphonuclear cell infiltration, and protease release. These contribute to tissue damage, cellular necrosis, and subsequent functional impairment (3, 4). In particular, the protease enzymes that release from both neutrophils and injured cardiac cells, could potentially aggravate the injury (5). In cardiomyocytes, protease enzymes are basically active, but their actions are regulated by transcription, translation, chaperone molecules, and endogenous inhibitors (2). In I/R injury, these regulatory mechanisms are altered, resulting in an increase in both intracellular and extracellular protease activities (6-11). The proteases present in the myocardium that are known to be involved in I/R injury, include calpain, matrix metalloproteinases (MMPs), and cathepsins (12). In particular, after reperfusion, inflammatory cell infiltration in the injury area led to increased injury area and intracellular structure change by enhancing protease enzyme activity and

chymase (13). Interestingly, this enzyme's effect on intracellular changes more than angiotensin converting enzyme (ACE) approximately 20-fold (14), resulting in contributing to the progression of heart failure by expanding cell injury, cell death, and cardiac remodelling. The inhibition of protease enzyme activities could be considered as a strategy to prevent ischaemic/reperfusion injury (13). Gene expression profiling revealed the 8,734 genes in response to cold ischaemia (CI) and reperfusion (15). Among the identified genes, secretory leukocyte protease inhibitor (SLPI) was thereby identified as a particularly promising candidate for treatment or prevention of I/R injury and post-ischaemic inflammation (16), as well as I/R injury in transplanted heart (17).

Secretory leukocyte protease inhibitor (SLPI) is a 107-amino acid-long protein in the whey acidic protein family (WAP). SLPI is a 12 kDa non-glycosylated cationic protein that is described as an inhibitor of serine proteases such as neutrophil elastase, cathepsin G, tryptase, and chymase (18, 19). The SLPI play roles in various principle areas including antimicrobial agent, controls the processing of inflammatory mediator and protect the host from excessive tissue damage by proteolytic enzyme that release during inflammation, suppress the inflammatory response through transcription factor NF-κB and control the cell growth cell differentiation and cell apoptosis (20). On the other hand, SLPI can inhibit bacterial and fungal growth and viral infection (21). The previous studies showed that overexpression of human secretory leukocyte protease inhibitor (hSLPI) and treatment with recombinant human SLPI (rhSLPI) provided cardioprotection against an *in vitro* and an *ex vivo* I/R injury (22, 23). It is noteworthy that the cardioprotective effect of SLPI found in those studies was the results from a pre-treatment experimental model. However, the roles and mechanisms of rhSLPI and the consequences in post-ischemic and reperfusion periods, especially in an *in vivo* model have not been studied. Moreover, the cardioprotective effect of SLPI probably be interpreted in the way that SLPI inhibit proteases. Interestingly, the findings from previous studies showed an anti-protease independent effect of SLPI that suppress macrophage response to bacteria lipopolysaccharide (LPS), attenuate caspase-3 activation and DNA degradation through inhibition TNF-α expression (24, 25). However, the requirement of anti-

protease activity of SLPI as cardioprotective agent against ischaemic and reperfusion injury, especially in an *in vivo* model, has never been investigated.

Therefore, this PhD dissertation aims to determine the post-ischemic roles and mechanisms of SLPI and the effect of anti-protease independent SLPI in an *in vivo* experiment. The outcome of this thesis was to provide more clinically relevant information about the therapeutic effect of SLPI, which strengthenedthe possibility of using SLPI as a therapeutic agent.

Objectives of this study

1. To investigate the effect of recombinant human SLPI on physiological and biochemical function, in rats subjected to *in vivo* ischaemia/reperfusion
2. To determine the anti-protease involvement in cardioprotective mechanisms of recombinant human SLPI against ischaemia/reperfusion

Hypothesis

Aim 1 To investigate the effect of recombinant human SLPI on physiological and biochemical function, in rats subjected to *in vivo* ischaemia/reperfusion

Hypothesis 1: post-ischaemic treatment of rhSLPI improves cardiac function and reduces infarct size in rat heart subjected to *in vivo* ischaemia/reperfusion.

Hypothesis 2: post-ischaemic treatment of rhSLPI reduced I/R induced p38 MAPK activation, activate pro-survival kinases and reduce apoptotic regulatory protein.

Aim 2. To determine the anti-protease involvement in cardioprotective mechanisms of recombinant human SLPI against ischaemia/reperfusion

Hypothesis 1: SLPI increase cell viability and reduce intracellular ROS level in H9c2 cell that subjected to *in vitro* H/R condition in anti-protease independent manner.

Hypothesis 2: SLPI improves cardiac function and reduces infarct size in rat heart subjected to *in vivo* ischaemia/reperfusion in anti-protease independent manner. And apoptotic regulatory proteins.

Scope of the study

The study was determining the effect of secretory leukocyte protease inhibitor (SLPI) and antiprotease deficiency SLPI in ischaemia/reperfusion (I/R) injury *in vivo*. The experiment is divided to 2 parts including

1. To determine the effect of post-ischemic treatment of recombinant human SLPI. An *in vivo* experiment, adult male Wistar rats were used as experimental animal model. The surgery for ischaemia/reperfusion (I/R) injury was generated on left anterior descending (LAD) artery. The recombinant human SLPI was administrated to the rats via intrajugular vein route in different timing including during ligation (DI) and onset at reperfusion (OR). The cardiac physiology was measured and recorded along with the surgery via pressure catheter that canulated through jugular vein and located in left ventricle (LV). At the end of surgery, infract size and area at risk were evaluated by Evan blue and TTC staining. Moreover, blood serum and cardiac tissue were collected for analysis for cardiac injury markers were measured from the blood serum. The cardiac tissues were homogenated and measured apoptotic regulatory protein by Western blot analysis.

2. To determine the cardioprotection effect of antiprotease-deficiency of SLPI, the cardiac myoblast cell line (H9c2) was used for an *in vitro* experiments. The antiprotease-deficiency form of SLPI, generated by site directed mutagenesis by Professor Cichy, was purified and used in this study. Cellular toxicity of anti-protease deficiency mutant SLPI was evaluated in H9c2 cell via MTT assay. The intracellular ROS was measured in H9c2 subjected to an *in vitro* hypoxia/reoxygenation (H/R). For an *in vivo* experiments, adult male Wistar rats were used as experimental animal model. The open-heart surgery and left anterior descending (LAD) artery was performed for simulating myocardial ischaemia/reperfusion (I/R) injury. Rats were treated with recombinant anti-protease deficiency mutant SLPI, as well as wildtype SLPI, at onset reperfusion timing while recording left ventricular pressure (LVP) parameters as cardiac physiology via pressure catheter located in left ventricle. The

infarct size and area at risk , ventricular pressure was determined as physiological parameters. Moreover, blood serum and cardiac tissue were collected for analysis of cardiac injury markers. The cardiac tissues were homogenated and measured apoptotic regulatory protein by Western blot analysis.

CHAPTER II

LITERATURE REVIEWS

1. Cardiovascular diseases

Cardiovascular diseases (CVD) are a group of heart and blood vessels disorders including coronary heart disease, cerebrovascular disease, rheumatic heart disease and other condition. The report from World Health Organization (WHO) address that CVD is leading to cause of the death globally, which taking estimated 17.9 million people, especially heart attack and stroke were accounting to 32% of global death in 2019 (26). The majority of death was found in prematurely people which under 70 years of age. Similarly, the information of world heart federation also shows that CVD is the world's number 1 killer that cause of 18.6 million deaths every year or about 33% of all global death as showed in Figure 1(27). Almost of CVD deaths take place in low and middle-income countries. The most important risk factors of CVD are the unhealthy behaviours including tobacco use, unhealthy diet, obesity, physical activity and harmful use of alcohol. Because of the most common cause of CVDs is a built-up of fatty acid plaque on the inner blood vessel wall that supply the heart and brain, American Heart Association(AHA) and National Institutes of Health suggests the way to keep the heart healthy is to be active, keep a healthy weight, learn about cholesterol, do not smoke and use smokeless tobacco and learn about sugar and diabetes (28).

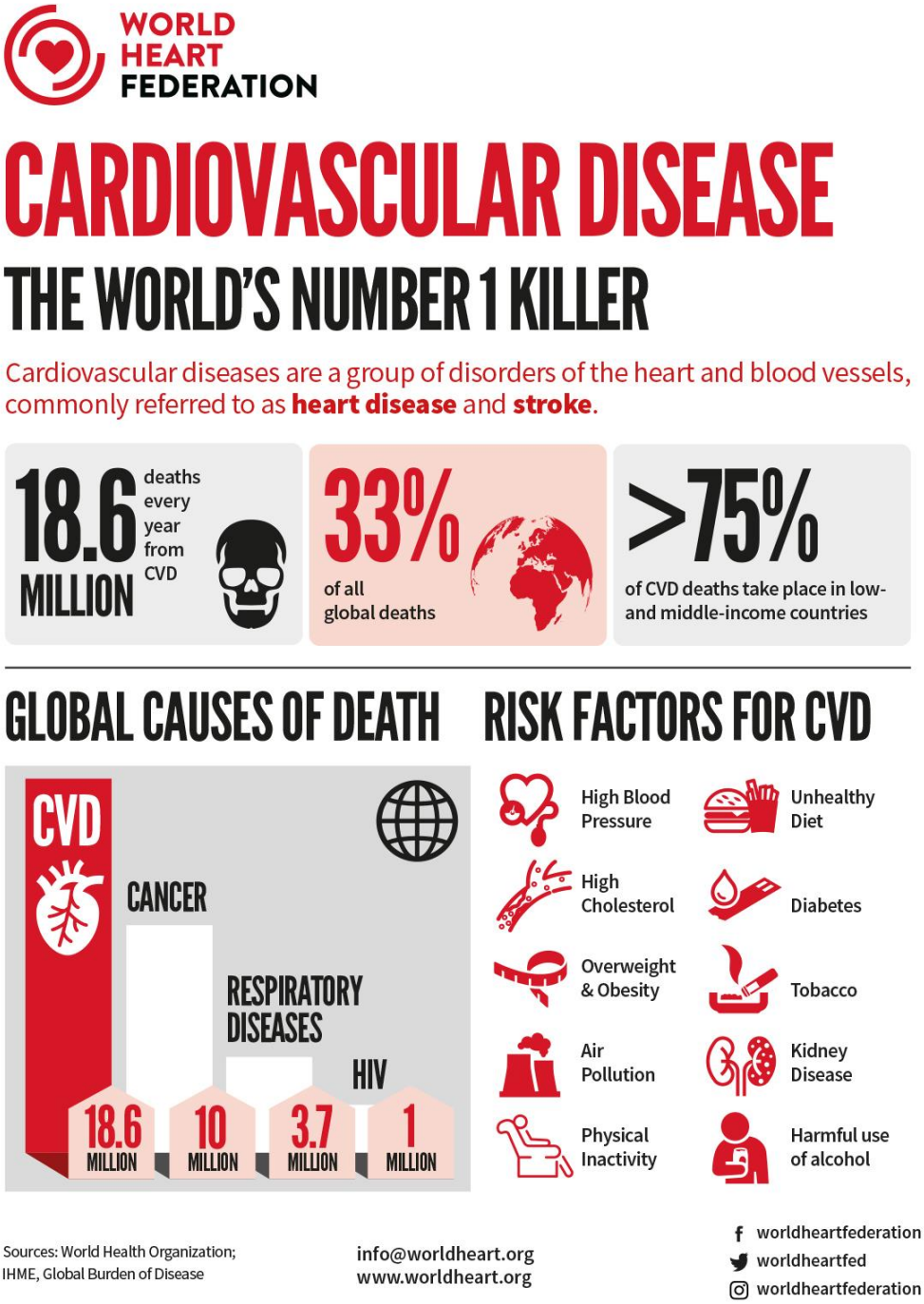


Figure 1 The information of heart disease and stroke statistics from World Heart Federation (27).

1.1 Definition of myocardial ischaemia

Myocardial ischaemia is cause from blockade of coronary artery, which is supply the blood and oxygen to heart tissues. The blockade of coronary artery decreased the perfusion of the blood to heart tissue that due to thrombosis, acute alteration of coronary atherosclerotic plaques. The result from loss of oxygen or blood supply to heart tissue not only imbalance oxygen supply and oxygen demand but also altered biochemical mechanism in the heart tissues, especially loss source of ATP production, oxygen, for energy metabolism. A lack of oxygen alters heart tissue to anaerobic metabolism for generate ATP that result in accumulation of hydrogen ions and lactate. The metabolic changes lead to irreversible injury in heart tissues (29). The procedure that reperfusing the blood to ischaemic area is called the reperfusion. The reperfusion aims to stabilize a plaque or prevent a disease progression in patient that was early diagnostic (30). On the other hand, the revascularization was performed in myocardial ischaemic patients which including percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG) surgery. These procedure aims to reperfusing the effective flow to the ischaemic area that could reduce and limit the expanding of infarct area. However, the reperfusion also has worst effect to heart tissue that result of the re-flow blood to the ischaemic area.

1.2 Ischemic/Reperfusion injury

The Blockade of coronary artery was leading to heart tissue lack of oxygen supply and induce cardiac cell death. The cardiac cell death was associated with time of ischaemia. The prolonged ischaemic time could increase infarct size until all of cardiac tissue as showed in red dash line in Figure 2. After reperfusion from various procedures such as angioplasty, thrombolytic therapy, coronary bypass surgery and cardiac transplant, these are reperfusing the blood to supply ischaemic area again. In the theoretical, reperfusing blood flow could limit the infarct size and cell damage immediately after the flow back to the infarct area as showed in green dash line in Figure 2. On the other hand, the reflow blood is not limit the cell injury and infarct size immediately but there are contribute to cardiac cell injury as called “ischaemic/reperfusion injury (I/R)” after the reperfusion as showed in black line in

Figure 2 (31). Therefore, I/R injury is common problem after various intervention such as angioplasty, cardiovascular surgery and cardiac transplantation (32).

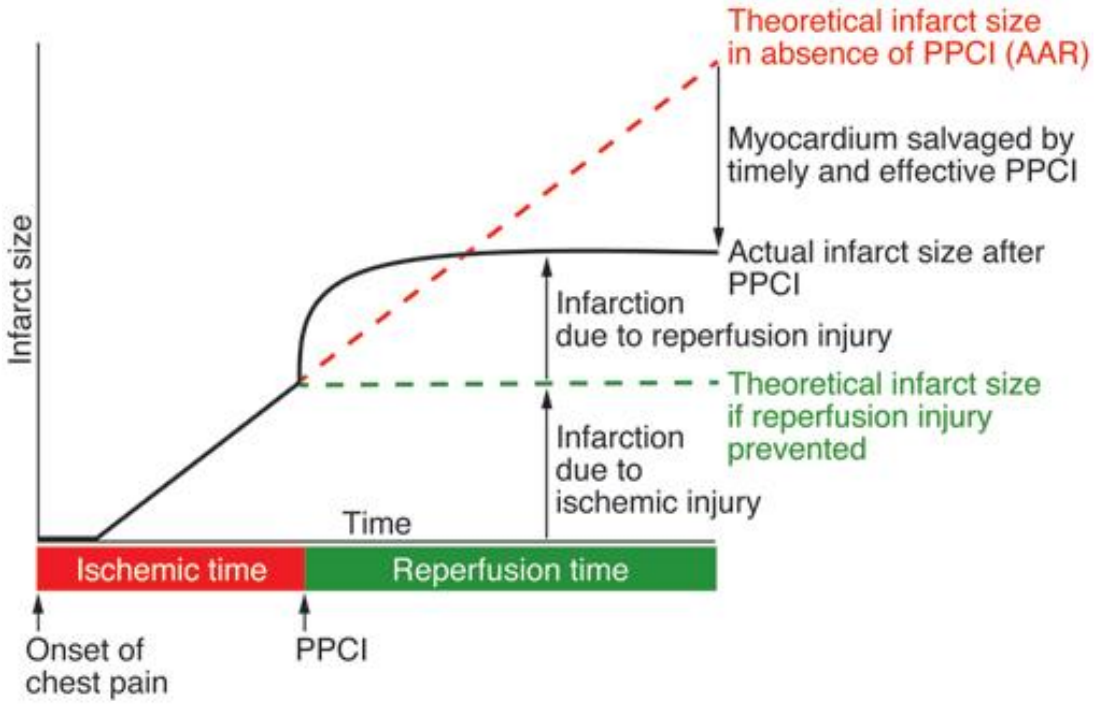


Figure 2 The concept of lethal reperfusion injury. The infract size are associated with prolonged ischaemia time that increase infarct size as red dash line. After ischaemic, infract size are increase until the reperfusion performed. In theoretical, infarct size could limit immediately after performed the reperfusion as green dash line. Infarct, the infarct still increased after reperfusion and stop at the end as black line (33).

A few minutes after a reperfusion, ROS is generated by various source The reperfusion is generated ROS burst and develop intracellular Ca^{2+} overload that impairing the recovery of the cardiac function. Although, during myocardial infraction, pH is decreased less than 7.0 that lead to intracellular acidosis but in reperfusion periods, the pH rapidly restored by remove lactate and activate Na^{+}-H^{+} exchanger as well as Na^{+}-HCO^{-} symporter. These lead to rapidly shift the intracellular pH that contribute to cardiomyocyte death by cardiomyocyte hypercontracture as shown in Figure 3 (33). The ROS and Ca^{2+} overload can activate and modify the protease activity in the cell. The activation of various protease activity in the heart promote the development of cardiac dysfunction after I/R injury (29).

Intracellular changes in I/R injury

I/R injury was consisting of various mechanism that including programmed cell death, activation of immune response system and intracellular oxidative stress. The numerous pathways and signalling were activated and affect to the heart tissue in both ischaemia and reperfusion duration.

During ischaemia, the beginning of cascade was the impairment of coronary artery that led to lack of blood supply to cardiac tissue. The area of infraction depends on the period that lack of perfusion. After occlusion, the infract begin in the endocardium and progresses transmurally overtime to epicardium as “wave front” so the duration of occlusion is related to the infarct size (34). Moreover, the ultimate size of myocardium cause from coronary occlusion as called “area at risk” (34). Lack of blood and oxygen supply contribute to stop mitochondrial oxidative phosphorylation from loss the oxygen that is major source to generate the ATP for energy metabolism. Anaerobic glycolysis metabolism is occurred instead of aerobic glycolysis metabolism for generate the energy (Figure 3).

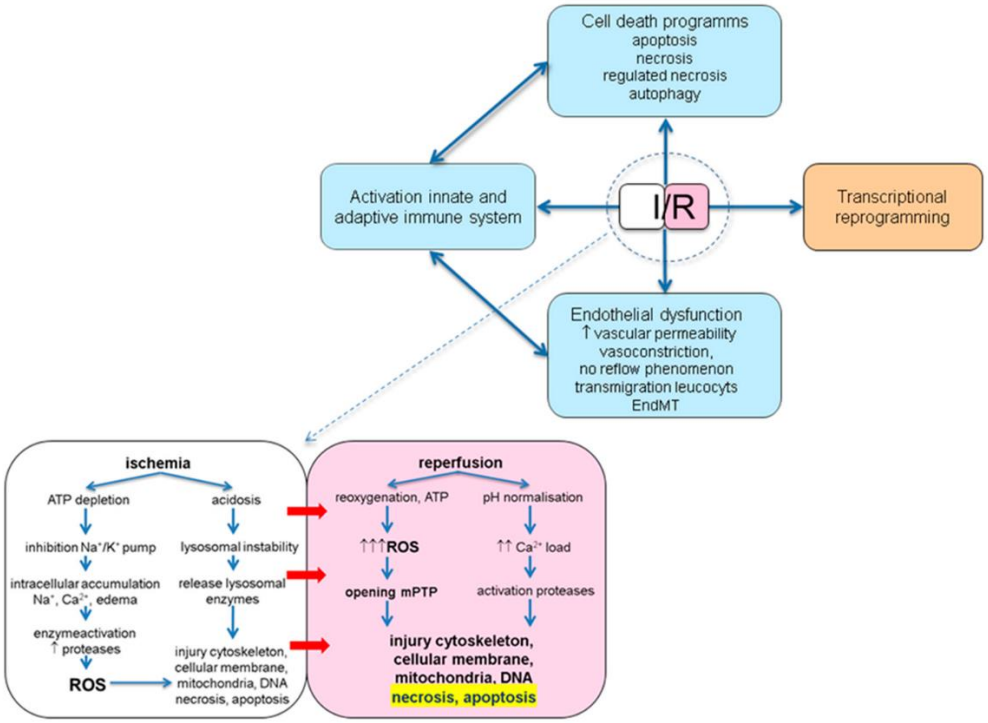


Figure 3 Schematic illustrating cellular change during ischaemia (13)

The major product of anaerobic glycolysis is hydrogen ion and lactic acid that lead to intercellular acidosis (5). Fatty acid is used in mitochondria oxidative phosphorylation instead of glucose. Moreover, the decreasing of intracellular pH leading to inhibit the activity of enzyme in the cell, so the cell needs to remove hydrogen ion from the cell for improve intracellular pH. The hydrogen ion can efflux from the cell by Na^+, H^+ exchanger (NHE) that remove hydrogen ion from the cell exchange with Na^+ ion. Na^+ influx is contributed to water influx into the cell and lead to cell swelling. In addition, the less energy and ion change also impair the excitation contraction uncoupling and electrical activity. Initially, electrical activity impairment cause from lack of ATP for Na^+, K^+-ATPase, which result to Na^+ and Cl^- influx into the cell. The accumulation of Na^+ ion enhance the influx of water into the cell and lead to cell swelling. Moreover, increasing of K^+ efflux from the cell that contributed to ventricular arrhythmia. The decline of ATP is not affected only Na^+, K+-ATPase. Ca^{2+} ATPase (SERCA) also affects from lack of ATP that used ATP for uptake Ca^{2+} ion into sarcoplasmic reticulum (ER) leading to accumulation of Ca^{2+} ion in ER, Ca^{2+} overload so the Ca^{2+} efflux might also regulate by Na^+/ Ca^{2+} exchanger (NCX) and plasma-membrane Ca^{2+} ATPase (PMCA). The accumulation of Ca^{2+} ion is contributed to ER stress. However, the intracellular Ca^{2+} overload lead to cell death and cell injury (29, 35) (Figure 4). Mitochondria can uptake Ca^{2+} ion by uniporter transporter but size of mitochondrial Ca^{2+} pool is small in normal condition. However, Ca^{2+} also influx and accumulated in mitochondria under pathological condition (5). The accumulation of Ca^{2+} ion in mitochondria can release from different way including reversal uniport transporter permeability of transition pore (PTP) and Na^+/H^+ dependent Ca^{2+} exchanger. Mitochondria is also needing to remove Ca^{2+} ion by Na^+/H^+ dependent Ca^{2+} exchanger, which remove Ca^{2+} ion exchange with influx Na^+/ H^+ ion. The influx of Na^+ ion induce water influx into the mitochondria that lead to mitochondria swelling and rupture (36).

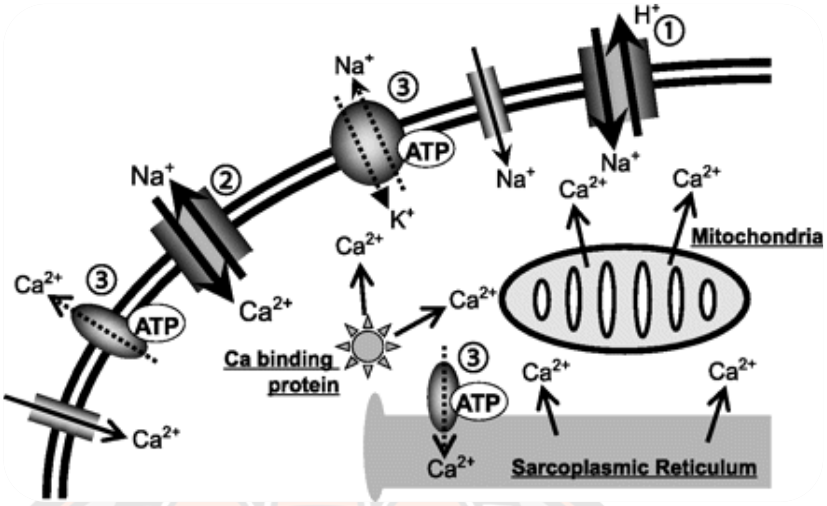


Figure 4 Ion exchanges during ischaemia: 1) excretion of H^+ due to pH lowering, 2) deactivation due to loss of ATP, and 3) reduction of Na^+/Ca^{2+} exchange due to lowered extracellular pH and intracellular accumulation of Na^+ (37).

Outer mitochondria membrane (OMM) rupture is results in mitochondria swelling that release pro-apoptotic mitochondria protein such as cytochrome c and Bcl-2 family proteins (36). Apoptosis is a generally encoded program leading to cell death both of normal development and homeostasis. The characteristic of apoptosis including cell shrinkage, nuclear fragmentation, chromatin condensation and membrane blebbing. These are the result of caspase protease activity. The apoptosis can activation through 2 main pathway including intrinsic and extrinsic pathway that can activate through B cell lymphoma 2 (Bcl-2) protein family both of anti-apoptosis Bcl-2 proteins and pro-apoptotic proteins such as Bcl-2, Bcl-2-associated X protein (BAX) and Bcl-2 antagonist or killer (BAK) (36). The intrinsic pathway can be activated from DNA fragment or ER stress, which is activate BAX and BAK. BAX and BAK activation lead to forming lipid pores on outer OMM and releasing cytochrome c from the intermembrane space (IMS) (38). The releasing of cytochrome c can bind apoptotic protease activating factor -1 (APAF1) that induce changing a conformation of caspase activation platform as apoptosome (39). The recruitment and dimerization of apoptosome activate initiator apoptotic caspase, caspase 9. Caspase 9 cleaved and activated executioner apoptotic caspase, caspase 9 and caspase 7, which lead to cell apoptosis (40). On the other hands, extrinsic pathway can activate by death ligand binding to death receptor, which induce recruitment and dimerization of adaptor protein, FAS-associated death domain protein (FADD). FADD activate initiator caspase, caspase 8 that cleave and activate the executioner caspase, caspase 3 and caspase 7. Then, caspase 3 and caspase 7 can activate a cell apoptosis. In addition, caspase 8 is also activate BAX or BAK through Bcl-2 homology 3 (BH3) interacting domain death agonist (BID) (41)

Mitochondria balance cell apoptosis pathway by activate anti-apoptotic Bcl-2 protein for inhibiting BID and activation of BAX and BAK. Moreover, mitochondria release X-linked inhibitor of apoptosis protein (XIAP) that can inhibit caspase activity both of recruitment of caspase 9, activation of caspase 3 and caspase 7. Whereas, after activation initiator caspase, mitochondria release second mitochondria-derived activator of caspase SMAC and OMI for block the XIAP activity as Figure 5(36).

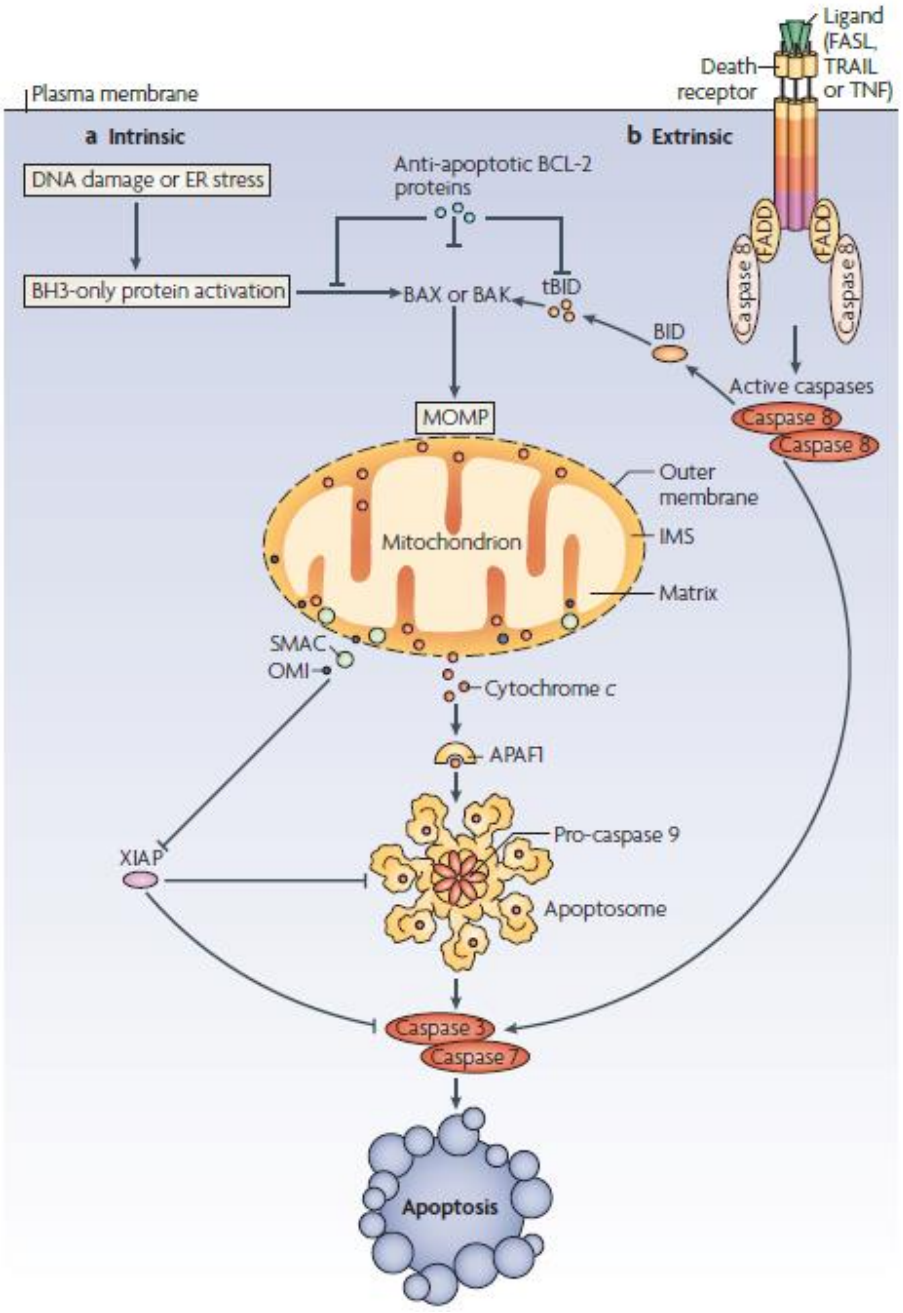


Figure 5 Intrinsic and extrinsic pathways of apoptosis (36).

However, intracellular Na^+ ions accumulation that led to programmed cell death by mitochondria swelling was not only affected from electrical ions imbalance during ischaemia. The other results of ion's imbalances that along with Na^+ ions accumulation was Ca^{2+} accumulation. Ca^{2+} ions accumulation was crucial factors that contribute to myocardial infarction progression because these accumulation led to the activation of Ca^{2+} dependent proteases enzyme such as calpains. Even though calpain still inactive in ischaemic period until normalization pH in reperfusion period and damage to the cells.

Although reperfusion restore blood flow and normalize pH level that contain oxygen and substrate to ischaemia area. The blood flow wash out accumulated H^+, normalize pH and delivery substrate for aerobic ATP generation (5) (Figure 3). Even reperfusion can reduce infract size by up to 50 % but reperfusion still be double-edge sword that cause cellular damage (42). Cellular damage from reperfusion was occurred underlying generation of reactive oxygen species (ROS). ROS is lead to calcium overload and opening of mitochondrial permeability transition (MPT), which release cytochrome C, succinate and mitochondrial DNA (mt-DNA). These substances were molecules that could induce cell death through apoptosis, necrosis and activated immune response as damage associated molecular pattern (DAMPs) (13).

1.5 Protease enzymes in I/R injury

Protein is important components of the cell functions. Proteases are the enzyme, which cleave the protein to be the amino acid. Protease can classified as serine, cysteine or threonine protease, aspartic, matrix metalloprotease based on their site of action (43). In pathophysiology of heart diseases is due to the function of various extracellular and intracellular protein, especially, the protease enzyme that cleaved peptide bond (10). The alteration of protease enzyme due to the mechanism underlying the myocardial I/R injury produce oxidative stress, Ca^{2+} overload and pH changing. These can cause altered functioning of various organelle in the cell such as extracellular matrix (ECM), sarcoplasmic reticulum (SR) and mitochondria that also leading to activate protease enzyme and impair contractile ability of the heart cell (10). The alteration of protease enzyme in cardiac tissue can lead to cardiovascular diseases (44). Protease is essential enzyme that play role in homeostasis of extracellular matrix (ECM) by degradation of misfold protein, malfunctional protein and protein turnover (45). The I/R injury increase a protease activity that led to changing of the structure and cell population both of intracellular and extracellular matrix depend on type of protease enzyme (46). The essential protease in the cardiac cell including calpain, cathepsin and matrix metalloproteinase (MMPs) as shown in Figure 6(2).

Calpain is a family of Ca^{2+} -dependent cysteine protease that involve in the proteins structure degradation and cell death pathway (2, 47, 48). Calpain is produced and localized in the cytosol as proenzyme that can be activated by intracellular Ca^{2+} influx (43). However, calpain can be directly activated by phosphorylation at serine 50 through ERK even without calcium influx (49). Family of calpain is including μ-calpain (calpain I), m-calpain (calpain II) and muscle specific calpain. The μ-calpain and m-calpain is highly expressed in the heart (48, 50). Calpain translocation can be induced by Ca^{2+} during ischaemia reperfusion. However, calpain activation only occurred after reperfusion that normalized intracellular pH because intracellular acidosis inhibits calpain activation during ischaemia (47). Moreover, calpain is known to contribute to cardiovascular diseases, since it involved in the degradation of troponin I (TnI), which is component of the actin tropomyosin complex during I/R injury (51). Treatment with specific calpain inhibitor, MDL-28170, could reduce

infarct size (47). Furthermore, the aldehydic calpain inhibitor, calpeptin, improved cardiac function such as ejection fraction (EF) after treated in mouse myocardial infarction model (48).

Cathepsins are lysosomal proteases family that primary protease enzyme found in acidosis environment of lysosome (43). Cathepsin are classified into serine (cathepsin A and G), aspartic (cathepsin D and E) and cysteine cathepsin depend on catalytic activities. Cathepsins is enzyme that function in acidosis environment (52). Therefore, I/R injury alter the phenotype of lysosome to lysosomal autophagy after 40 min of hypoxia that leading to mitochondria degradation (53). Moreover, cathepsin gene expression in the heart is enhanced by angiotensin II (54) However, cathepsin D and L are increase activity in I/R injury and activate MMP that cleavage fibronectin, laminin, type I, IV and XVIII collagen (55, 56).

The matrix metalloproteinase (MMPs) discovered to be crucial tools in tadpole morphogenesis (57). MMPs are endopeptidases that utilize a highly-conserved zinc-binding catalytic centre in order to cleave protein. MMPs can be divided into 5 groups depend on substrate preference including collagenases, gelatinases, stromelysins, matrilysins (58). MMP-2 and MMP-9 are enzyme for degrading type IV collagen that is important major component of basement membrane (58). The MMPs can be activated by high level of ROS during I/R injury (59). In addition, MMP-2 and MMP-9, gelatinase, are found in cardiac myocytes cardiac fibroblast and endothelial cell (60). MMP-2 and MMP-9 have been found increasing activity in myocardial I/R injury (60, 61). Moreover, MMP-2 and MMP-9 is play role in early and late of late matrix remodeling after myocardial I/R injury (61, 62). MMP-2 and MMP-9 are activated both in myocardium and plasma in acute myocardial ischemic/ reperfusion injury patients (60). Furthermore, MMP activity and ECM degradation can be regulated by chymase angiotensin converting enzyme (ACE) at post-translation level (63). Interestingly, the expression level of MMP is elevated in hypertensive models, especially, angiotensin II induced hypertension (64). Moreover, overexpression of MMP-1 is related to accumulation of cardiac fibroblast and cardiac dysfunction and remodeling due to chronic pressure overload (65). Besides, MMP-2 and MMP9 are upregulated in the dilated failing heart and development of cardiac remodeling model (66).

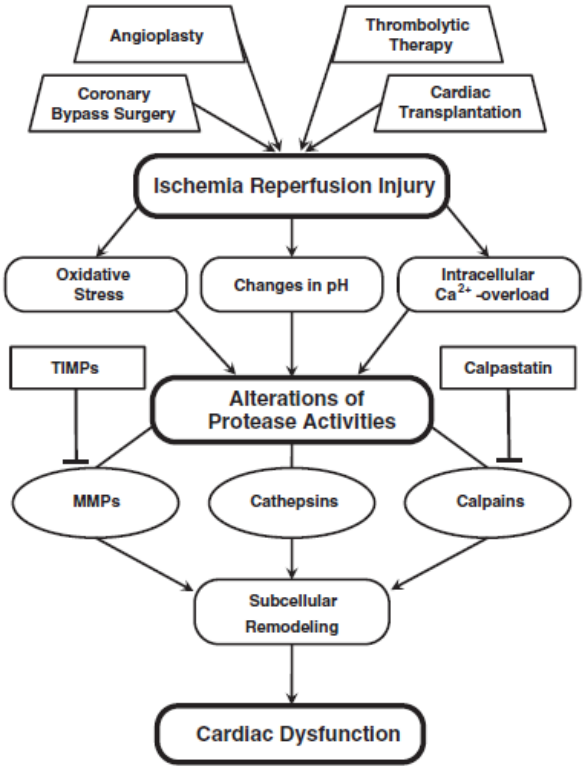


Figure 6 Schematic representation of the mechanisms of I/R induced changes in cardiac (2).

1.6 Inflammation and ischaemia/reperfusion injury

After cell injury in I/R injury, ROS was one of molecules that releasing in injury area. These have potential to directly injury the cells and trigger inflammatory cascade through the induction of cytokines. This activation led to 3 repair overlapping phases that including inflammation, reparative or proliferation and mature phase. Firstly, inflammation phase was the activation of chemokines and cytokines cascade that resulting in recruitment of local infiltration of leukocyte into the infarct area. Second, proliferation phase, mononuclear cell and macrophage was activated and released cytokines and growth factor, which recruit and active myofibroblasts and vascular cells. The activation of myofilament was released extracellular matrix protein that was overlap to last phase. Lastly, maturation phase, reparative fibroblast and vascular cells become apoptosis and cross-linked collagen-based scar was formed (67).

Inflammation in I/R injury is defined as sterile inflammation that the inflammatory response associated with the recognition of releasing endogenous and non-pathogenic signals from damaged cell as DAMPs (13, 68). The endogenous ligands is released from cell death that including high-mobility group box-1 (HMGB1), heat shock proteins (HSPs), S100-proteins and mitochondrial DNAs (mt-DNAs). The releasing of DAMPs activates transmembrane proteins, including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptor family of cytosolic proteins also known as Nod-like receptors, NLRP3 inflammasome, function as a pattern recognition receptors (PRR), especially TLR2 and 4 that upregulated in tubular epithelial cell in ischaemia (69, 70). The TLRs activation led to recruitment of downstream various adaptor molecules such as TNF-receptor-associated factor, myeloid differentiation primary-response protein 88 (MyD88) and toll-interleukin 1 receptor (TIR) domains that result to activation transcription factors consist of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and IFN-regulatory factor -3 (IRF3). These transcription factors regulate pro-inflammatory gene transcription and inflammatory response (71). In addition, NF-κB can also be activated by free radical, ROS, and cytokines including TNF-α and IL-1β (72). An important source of preformed and newly synthesized cytokines is mast cell degranulation that usually occurs in ischaemic area (73). The

result of degranulation of mast cell is a rapid release of TNF-α that induce IL-6 for mononuclear cells infiltration. So TLRs activation plays crucial role in trigger of the cytokines cascades and MAPK pathways (74). On other hands, non-pathogenic signals, released from damaged cell in I/R injury, lead to activation of NLRP3 inflammasomes, which are large multiple protein complexes (68). NLRP3 inflammasomes compose of 3 components including a member of the NLRP family proteins, NLRP3, apoptosis-associated speck-like protein, which consist of caspase recruitment domain (ASC) and pro-caspase-1 (75). The consequence of NLRP3 activation is IL-1β production that is converted by caspase-1. Then, IL-1β induces the initial pro-inflammatory response and release of other inflammatory cytokines that facilitate the recruitment and activation of inflammatory cells, especially neutrophils and monocytes (68).

The inflammation response to I/R injury is characterized by recruitment of neutrophils and other leukocytes in I/R site (76). The inflammatory response to tissue cause by production and secretion of cytokines, chemokines and proinflammatory cytokines (13). During reperfusion, leukocyte trafficking into ischemic sites by 11 steps (76) as shown in Figure 7 including 1) Margination, neutrophils from capillaries move into larger diameter vascular postcapillary venular segment of microvascular by hydrodynamic forces from blood flow to endothelial wall. 2) Tethering and rolling, neutrophil binding to adhesion molecule that express on activated endothelial cells during I/R injury. 3) Slow rolling, after leukocyte bind to adhesion molecule that enable leukocyte to slow rolling by forming more weak interaction with selectins on endothelial surface. Moreover, surface of leukocyte forming to long nanotubes for tethering and rolling leukocyte. 4) Firm or stationary adhesion, leukocyte develop strong adhesion by integrin-dependent interaction with endothelial ICAM-1 that rolling leukocyte and firming adhesion. 5) Luminal crawling, ICAM-1 activate intracellular signalling that change cytoskeleton and polarization of the cell. Crawling leukocyte move along endothelial cell for explore interendothelial junction that is route for diapedesis of leukocyte. 6) Transendothelial cell migration, leukocyte cross endothelial cell through interendothelial junction and overlaying on basement membrane that express low matrix protein (Pr^-) deposition. 7)Abluminal crawling, the

diapedesing leukocyte interacting with basement membrane structure by adhesion molecule. 8) Penetration of pericyte gaps and regions of low matrix protein expression in the basement membrane, abluminal crawling leukocyte break the layer at region of low matrix protein deposition in the basement membrane that leading to leukocyte can move through this barrier. 9 and 10) Detachment from the vessel wall and migration through the interstitial matrix, leukocyte migrate into the tissue space that due to detaching from basement membrane components and adhesion molecules. These lead to release pseudopodal extensions from basement membrane and migrate into tissue space as inflammatory foci. 11) Attachment to and attack on parenchymal cells, leukocyte release cytotoxic molecules such as ROS and hydrolytic enzymes that cause call injury (76).

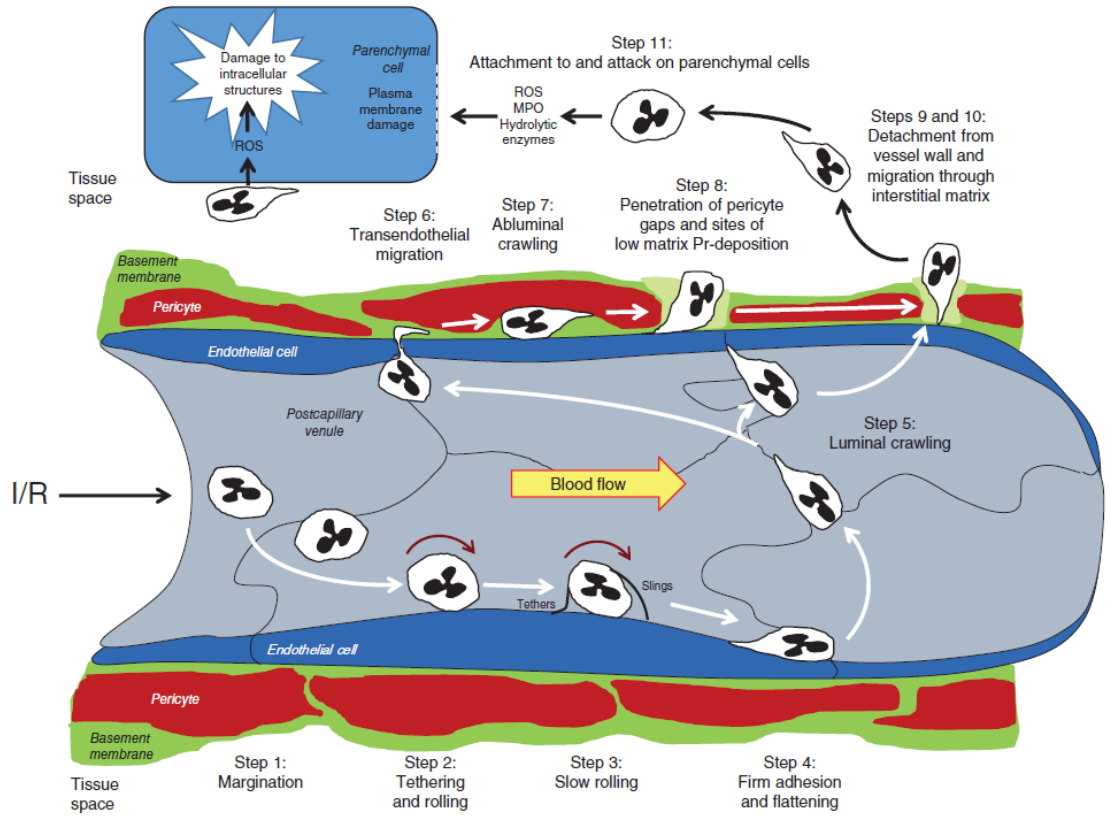


Figure 7 Neutrophil trafficking to ischemic sites occurs during reperfusion of ischemic tissues and involves 11 distinct steps (76).

The inflammation is a key defences mechanism in order to restrict the injured area. However, inflammation is double-edged sword contribute to the progression of the disease. Inflammation responses are different depend on diseases, but share some common mediators of inflammation such as interleukin 1β (IL-1β), tumour necrosis factor(TNF)-α or IL-6 (77). Moreover, the primary inflammatory cytokines including IL-1β and TNF-α activate intracellular signalling pathway that involved in I/R injury, activation of mitogen-activated protein kinase (MAPK) pathway through the Toll-like receptor (TLR)-4, activation through IL-1 receptor or TNF receptor (78). Finally, these activate transcription factors, nuclear factor kappa B (NF-κB), especially, TNF-α is potent activator of NF-κB that lead to releasing amount of inflammatory cytokines and recruiting neutrophil into injury area (79).

Inflammatory serine protease inhibitor

Previous studies showed the relationship between ischaemia/reperfusion injury and neutrophil activation that activated though free radicals, expression of adhesion molecules and inflammatory cytokines (80, 81). Serine proteases are a key of inflammatory response by activated leukocytes and mast cells (82). The attenuation of ischaemia/reperfusion injury could reduce neutrophil activity, free radical scavengers, lipid peroxidation inhibitors, protease inhibitors, leukocyte depletion and adhesion molecules expression inhibitors (80, 81, 83). Previous studies showed that ONO-5046 Na, which is Polymorphonuclear (PMN) elastase inhibitor, could reduce and block PMN elastase activity and limit the inflammatory cascade indirectly on I/R injury in heart transplantation (81). In addition, this inhibitor also attenuated I/R injury in lung and liver (84, 85). In others inhibitor on the same condition, I/R injury in heart transplantation, Sivelestat is neutrophil elastase inhibitor that also lower CK-MB, lactate and inflammatory cytokines level (83). Similarly, the reported from M. Kambe *et al.* showed Siveslestat, neutrophil elastase inhibitor, improve cardiac function after administrated onset at the reperfusion in I/R injury condition (86).

Not only neutrophil elastase inhibitor, other protease inhibitors also attenuated I/R injury. Matrix metalloproteinase inhibitor, MMP-2-inhibitor-NO-donor hybrid, could improve coronary flow and heart rate against I/R injury condition. Moreover, the improvement is associate with a reduction of tissue injury and MMP-2

activity (87). Likewise, Krzywonos-Zawadzka and colleagues study showed MMP-2, myosin light chain, and nitric oxide synthase inhibitors improved mechanical heart function by decreased the expression level of iNOS, eNOS and enhance NO synthesis (88). The study of inflammatory serine proteases (ISPs) inhibitor, administered after 30 minutes of ischaemia and 24 hours of reperfusion, showed a reduction in cathepsin G and chymase activity, when compared with control group. IPS inhibitors also reduced myocardial apoptosis, infarct size and improved cardiac function that against to I/R injury (89). The reported from Kolpakov *et al.* showed dipeptidyl peptidase I (DPPI), which is lysosomal enzyme that involved in the ISPs, knock out mice markedly reduce the activity of neutrophil- and mast cell-derived serine protease after 4-week post-MI, when compared with wild type mice. Moreover, lacking of DDPI also improved cardiac contractile function along with promotion of angiogenic cytokines such as vascular endothelial growth factor (VEGF) A and B (90). Determination of creatine kinase and myeloperoxidase activity in myocardium after administration of serine protease inhibitor, aprotinin, in MI rats' model showed that it effectively preserved myocardium from I/R injury, as reduce induction of cardiac myocytes apoptosis even after 24 hours of refusion. Aprotinin also inhibited polymorphonuclear leukocyte-induced myocardial injury from I/R injury (91). Not only neutrophil elastase, cathepsin G is also involved in cardiovascular diseases.

Chymase from mast cell is also important similar to cathepsin G in cardiovascular pathology, especially the activation of angiotensin II pathway that contributed to cardiac remodeling and heart failure (92). Previous study showed that chymase involve in plaque progression and instability in atherosclerosis. After treated with chymase inhibitor, RO5066852, the reduction of spontaneous atherosclerosis and accelerated plaque progression found in the thoracic aorta of apoE $^{-/-}$ mice (93). Similarly, NK3201 was treated 3 days before ligation, the result showed NK3201 was reduced chymase activity without altering Angiotensin Converting Enzyme (ACE) activity (94). NK3201 improved pressure develop and reduced mortality rate 17% (95). In hamster, chymase inhibitor, Suc-Val-Pro-Phep (OPh)2, was injected into thoracic cavity that result in transforming growth factor β1 lowering along with reducing density of mast cell. Therefore, chymase inhibitor could attenuate postoperative cardiac adhesion (96). However, the findings from computational study

revealed the putative off-targets for chymase inhibitors were identified through various structural and functional similarity analyses along with molecular docking studies (97, 98). Then, the unpredictable adverse effect of small molecules was concerned that lead to endogenous anti-protease peptide was be alternative choices. The endogenous inhibitor usually express and function in the body which could be safe and reliable.

Signalling in I/R injury

The consequences of reperfusion result in releasing of intracellular death ligands, which induce various intracellular pathways including mitochondrial-mediated apoptosis (99). These transduces the signalling through broad spectrum of death stressors and death receptor that response to the releasing of external stimuli. However, mitochondria-mediated apoptosis is not only pathway that is activated after the opening of the mitochondrial permeability transition pores (mTTP) at the reperfusion time (100). Death receptors signalling, which is induced by releasing death ligand, can promote cell apoptosis pathway and cell survival pathway that enhance cardioprotection pathway. Cell survival pathway is consisting of reperfusion injury salvage kinase (RISK) pathway and survival activating factor enhancement (SAFE) pathway (101). This pathway is regulated by specific ligand binding to cell surface receptors. The important death receptor is members of the tumour necrosis factor (TNF) superfamily that consist of Fas, TNFR1, TRAIL2 (102). The signalling is activated from integral plasma membrane protein binding to FasL, which activate through Fas receptor. Interestingly, Fas receptor is usually express in all type of the cell type but basal FasL expression level is restricted to specific cell types (103). However, Jeremias *et al.* study reported that during ischaemia/reperfusion injury FasL is induced to express in the heart (104). In death receptor details, previous study describes the interaction of ligand and receptor is mediated by homotypic interaction of death-fold motif that is generally binding to the same motif type. Death-fold motif is several varieties of death domain consist of death domains (DD), death effector domain (DED), caspase recruitment domains (CARD) and pyrin domain (PYD). The interaction between TNF-α and TNFR could induce complex I assembly on the cytoplasmic tail of receptor. Downstream of complex I are including cell survival,

apoptosis through TRADD and RIPK1 recruitment. TRADD and RIPK1 recruitment are result in adaptor protein TNF receptor-associated factor 2 and 5 (TRAF2/5) and cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2) that attach to lysine 63 (K63)-linked ubiquitin chains. Ubiquitin chains promote the linear ubiquitin chain assembly complex (LUBAC) recruitment that also activate transforming growth factor-β-activated kinase 1 (TAK1) binding proteins 2 and 3 (TAB2/3). TAK1 play crucial role in inhibitor of κB kinase (IKKs) through NF-κB upregulation that leading to promote cell survival and inflammation. In addition, TAK1 activation also activate mitogen-activated protein kinase (MAPKs) pathway that provide additional survival pathway (99).

Mitogen-activated protein kinase are a family of heterogeneous serine/threonine kinase, which regulate cell growth, cell proliferation, survival and cell death (76). The intracellular signalling including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs. Furthermore, the all-MAPK pathways are consisting of protein kinase families including MAPK kinase kinases (MKKKs or MAPKKK), MAPK kinases (MKKs or MAPKKs) and MAPKs. MAPKs are activated by dual phosphorylation at tyrosine and threonine residue in a conversed Thr-*Xaa*-Tyr (Xaa is any amino-acid) in the activation loop of kinase subdomain VIII. MAPKs can reverse phosphorylation and return to inactivated state. MAPKs is specific catalysed by dual phosphorylation both of Ser/Thr residues that interaction between N-terminal region located on the MAPKKs (105). Cellular stress and cytokines can activate p38 through MAPKKKs, MAPKKs and p38 respectively that leading to cell adaptation and physiology response as shown in Figure 8 (106, 107).

The p38 MAPK was first identified in *Saccharomyces cerevisiae* as protein product of Hog1 (108). The mammalian p38 MAPK consist of 4 isoforms including α, β, γ and δ. The isoform that expresses in the most the cell types is p38α, which is 38 kDa polypeptide. However, other isoforms were identified by different encoded gens p38β and p38γ (as called SAPK3 and ERK6), which express in skeletal muscle and p38δ (as called SAPK4) that express in testis, pancreas, kidney and small intestine (109). Most stimuli that activate p38 MAPK can also activate JNK. Upstream kinases of p38 MAPK include MEK3 and MEK6, which is activated by upstream MAPKKKs from physical, chemical stress stimuli, inflammatory cytokines such as IL-1 and TNF-α (110). The MEK3/6 can activate p38 MAPK by the phosphorylation at Thr-Gly-Tyr (TGY) in activation loop that specific to p38 MAPK because length of activation loop is different from ERK2 and JNK (111). The p38 MAPK activation could also be activated in leukocytes by various cytokines or extracellular mediator, especially, respiratory burst or apoptosis (112). Moreover, p38 MAPK activation in macrophage and neutrophil are also regulate T-call differentiation and apoptosis through gamma interferon production (113) as same as in I/R injury. Since those stimuli can activate releasing of ROS, disrupt mitochondria membrane and activate inflammatory response that activate p38 MAPK, JNK and ERK (114). The downstream substrate of

p38 MAPK including MAPKAP-K2 or MAPKAP-K3 (115). These substrates of p38 contribute to subsequent phosphorylation to HSP27 that prevent actin cytoskeleton for cell migration (116). Moreover, these substrate can activate subsequent phosphorylation such as transcription factor ATF2 or SAP1 (107). In addition, p38 MAPK is involve cell death through activated translocated of Bax from cytosol to mitochondria (117). Not only play role in cell death, p38 MAPK have been found to be involved in cell growth, cell differentiation and cell cycle that activated by granulocyte macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor (FGF), erythropoietin, IL-3, IL-2, IL-7, insulin like growth factor (IGF), vascular endothelial growth factor (VEGF) (118-120).

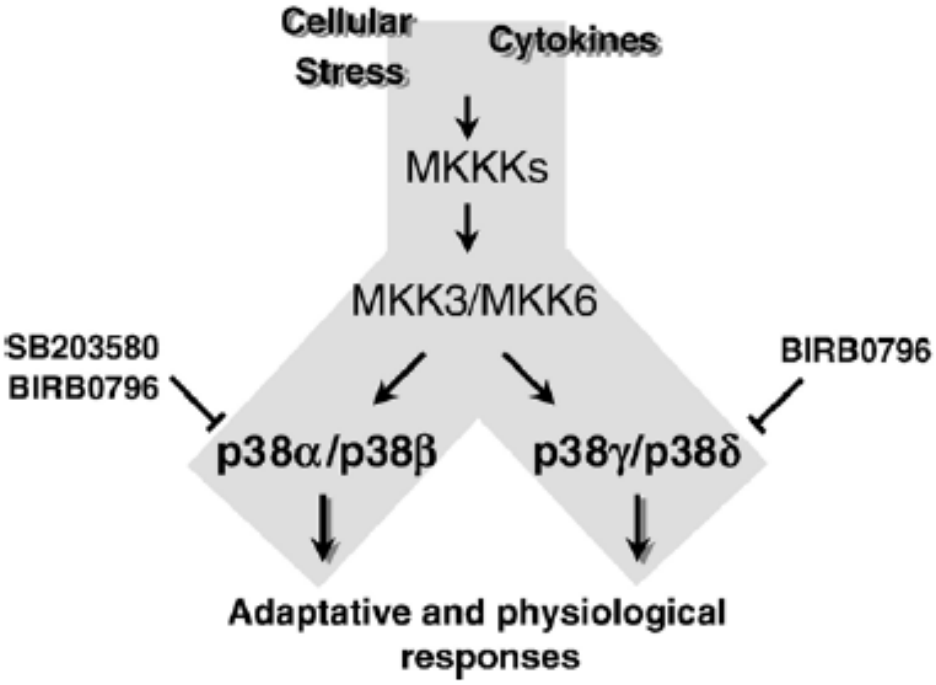


Figure 8 p38 MAPKs signalling pathways (107)

The c-Jun N-terminal kinases (JNKs) were firstly isolated from rat liver that injected with cycloheximide (121, 122). JNKs is activated from G protein-couple receptors, serum and growth factor (122). In mammals, there are 3 different isoforms of JNKs including JNK1, JNK2 and JNK3, which located on 3 different chromosomes. JNK1 and JNK2 are mostly express form that found in various tissues,

but JNK3 is mainly express in central nervous system (CNS) neurons, cardiac smooth muscle, and testis (123). JNKs localize from cytoplasm to the nucleus following stimulation. JNK activation, similar to ERK1/2 and p38, require dual phosphorylation on tyrosine/threonine residues within Thr-Pro-Tyr (TPY). JNK is also recognized to response to cytokines such as TNF-α and IL-1 (124). JNK signalling play major role in cell apoptosis (125). The signalling start from phosphorylation of MEK4 and MEK7 by MAPPKs such as MEKK1-4, MLK2, TAK1 and ASK1 and 2 (122). The substrate of JNKs is N-terminal of transcription factor c-Jun that phosphorylated on Ser63 and Ser73. JNK can also phosphorylate several transcription factors such as ATF-2, nerve growth factor (NGF), NFAT and STAT (110, 122). Furthermore, JNK can phosphorylate Bcl-2 and Bcl-X_L that is pro-apoptotic activity (126). Previous study showed p-JNK expression level was significantly increased in myocardial injury induced by I/R (127). The overexpression of tumour necrosis factor receptor associated factor -1 (TRAF1) was upregulates of JNK/p38 cascade that aggravates the development of myocardial I/R injury through inflammation and cardiomyocyte death (128).

On the other hand, the activation of pro-survival protein that reduce cell death through inhibit pro-apoptotic cascade against I/R injury by pro-survival proteins called as “Reperfusion injury salvage kinase (RISK) pathway. RISK pathway was first described by Yellon’s group in 2002 that showed cardioprotective effect underlying urocortin induction (129). The RISK pathway is combination of two parallel cascades including PI3K and ERK1/2. The RISK pathway has importance concepts that described function of this pathway. Firstly, short term activation of kinases is protective, the activation of pro-survival kinases are protective effect when acutely activation, whereas chronic activation could be consider to be enhance cardiac dysfunction (101, 130). Previous study reported that the alteration of these kinases’ activation was regulated by PTEN activation. PTEN was described as a crucial “switch” that regulated these pathway (131). Second concept, this pathway should be activated at the time of early reperfusion for cardioprotective effect. Previously, RISK pathway was demonstrated by activating at two time point underlying ischaemic preconditioning, which including “trigger phase” and “during the onset of reperfusion”. Trigger phase was during the preconditioning cycle at ischaemic

episode. During the onset of reperfusion was early phase of perfusion that related to the opening of mitochondrial permeability transition pore (mPTP). The target of protective effect is activate the pro-survival kinase within the first 15 min of opening mPTP when onset the reperfusion because these was loss the cardioprotective effect activation after 15 min (132). Therefore, common feature of cardioprotective effect is early activation of survival kinase signalling cascades including phosphatidylinositol-3-OH kinase (PI3K)-Akt and p42/44 extracellular signal-regulated kinase (ERK1/2).

Protein kinase B (PKB)/Akt was firstly discovered by three researcher groups in 1991 that reported PKB linked to cell survival pathway (133-135). PKB is activated downstream of phophatidylinositol-3-OH-kinase (PI3K) signalling cascades though wide range of receptors (136). The phosphorylation of PI3K results in lipids phosphorylated at the 3'-OH position of the inositol head group including predominantly phosphatidylinositol 3,4,5 trisphosphate (PIP_3) and ($PI_{3,4}$)P_2. The activation cause PKB translocated to plasma membrane. Phosphatidylinositol-3-OH-kinase (PI3K) participates in protection against ischaemia/reperfusion injury through serine/threonine kinase, Akt (137). The activation of PI3K/Akt is both of directly phosphorylate the pro-apoptotic protein BAD or indirectly phosphorylate and recruit of distal signalling moieties such as 70-kDa ribosomal protein S6 kinase (p70S6K), p90 ribosomal S6 kinase (p90RSK) (138, 139). The result of BAD phosphorylation is inhibited apoptosis by binding to 14-3-3 sequesters of mitochondria. Moreover, the PI3K/Akt activation is also inhibit the change of BAX conformation that require in translocation to mitochondria as in Figure 9 (140). The consequence of PI3K phosphorylation is a removal of various pro-apoptotic proteins such as BAD, BAX, and p53 from mitochondrial action site. Akt phosphorylation is also inhibit the opening of mPTP that leading to cytochrome *c* releasing from mitochondria and maintain mitochondrial membrane potential by inducing eNOS activation (141).

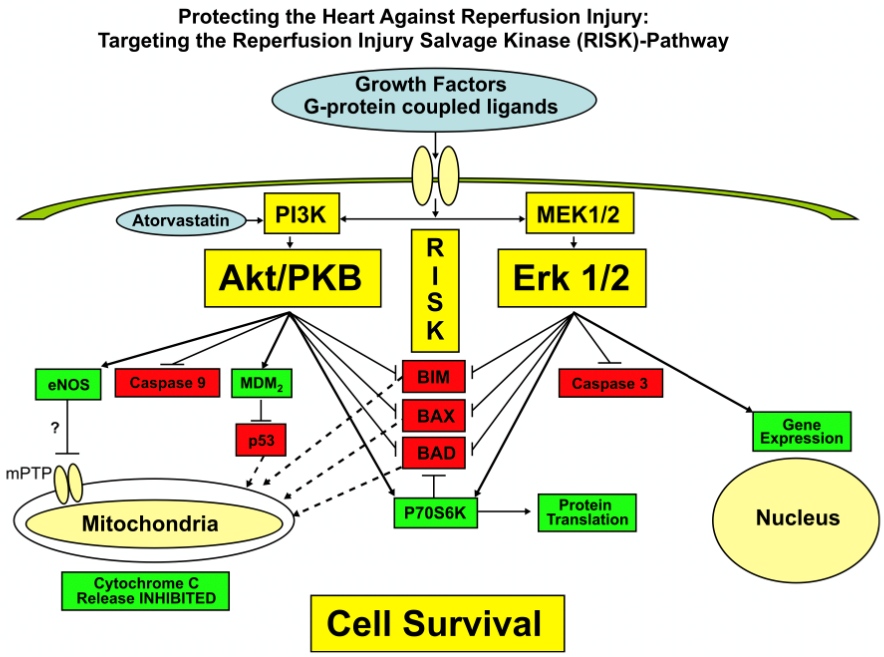


Figure 9 Targeting the reperfusion injury salvage kinase (RISK) pathway (140).

Extracellular signal-regulated kinases (ERKs) are serine/threonine protein kinase, which is classical mitogen kinase cascade including MAPKKKs A-Raf, B-Raf and Raf-1, MAPKKs MEK1 and MEK2, and MAPKs ERK1 and ERK2. ERK1 and ERK2 share 83% amino acid identity and express in various tissues (110). ERK1/2 has relative molecular weight of 42/44 kDa. ERKs is normally located in the cytoplasm. Intracellular and extracellular can induce serine/threonine phosphorylation in the VII region of ERK1/2. The regulation of ERK1/2 is important for maintenance of cell proliferation, growth, development, and differentiation. ERK1/2 is activated by ligand of the heterotrimeric G protein couple receptor and cytokines. Cell surface receptor, tyrosine kinase (RTK) and G protein-coupled receptor activate signals through Raf/MEK/ERK cascade through GTP-binding protein Ras. Active Ras or Ras-GTP transmit signal to downstream effector proteins, serine/threonine kinase Raf. Ras-GTP binds to Raf induce its translocation from the cytoplasm to the cell membrane. Translocation generates transient membrane-associated signal. The activated Raf bind and phosphorylate dual kinase MEK1 and MEK2, which subsequently phosphorylate ERK1/2 within Thr-Glu-Tyr (TEY) in activation loop. Only 5% of activated Ras molecules can induce full ERK1/2 activation (142). The ERK1/2 activation is crucial for translocation into the nucleus, which then subsequently phosphorylates various transcription factors such as STATs, ATF_2, Elk-1, c-Myc,Fos and Jun. In I/R injury, the heart damage can lead to irreversible fatal injuries and death (143). Previous study showed the relationship between ERK1/2 signalling pathway and inflammation in I/R injury (144). The activation of ERK1/2 could downregulate inflammatory cytokines in ameliorates acute myocardial infarction in rats (145). Moreover, ERK1/2 activation was decreased myocardial inflammatory cytokines including TNF-α and IL-1β that related to reducing of infarction size and releasing of cardiac troponin I (cTnI) through upstream master switch ,upregulating early growth response (Egr)-1 (146).

Oxidatively modified protein: Protein carbonylation (PC) and ischaemia modified albumin (IMA) as a marker for I/R injury

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) and antioxidant molecules (147). ROS is produced in normal physiological condition that play role in various enzyme cascades in cellular metabolic processes. The source of ROS is mitochondria that produce superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) (148). These ROS can form through electrons leakage from electron transport chain. ROS is important initiator and mediator of many diseases, especially, cardiovascular diseases (149). The continuous and cumulative of ROS is damage both of directly and indirectly all biomolecules including proteins, liquid, DNA and carbohydrate (150). Among biomolecules that oxidatively modified, biomolecule carbonylation is major hallmark of oxidative stress (151). Protein carbonylation was firstly described in 1990 by Levine and Stadman (152). It is recognized as universal marker of oxidative stress that linked to human diseases such as Alzheimer's disease, chronic lung disease, chronic renal failure, diabetes sepsis and ischaemia/reperfusion injury (153). Protein carbonylation is irreversible post-translational modification (PTM) that cause from ROS oxidized amino acids and protein carbonyl groups (154). The products of oxidation, reactive carbonyl group, including high molecular weight (HMW) carbonyl and low molecular weight (LMW). HMW is including aldehyde, keto and lactam groups formed in the targeted biomolecules which is carbonylated phospholipids, proteins and DNA. On other hand, LMW caused by oxidative cleavage reactions, methylglyoxal derived from protein glycoxidation and lipid peroxidation. The accumulation of these product is characteristic of carbonyl stress that is nucleophilic substrates and leading to alteration of biomolecules. Alteration of biomolecules were increase cell toxicity that lead to cell apoptosis (155). Previous studies showed protein carbonyl level was related to the oxidative stress in the cardiovascular diseases. The muscle ischaemia reperfusion injury in rats showed the protein carbonylation level was significantly increased in I/R group that related to xanthin oxidase (XO) and adenosine deaminase (AD). In intermittent hypoxia on oxidative stress induced myocardial damage in mice study showed an increasing in protein carbonyl related to lipid peroxidation an infarct size in intermittent hypoxic injury (156). In addition, plasma protein carbonyl

concentration is also increased in left ventricular hypertrophy (LVH) patient when compared to patient with normal geometry (154). The correlation of protein carbonyl to ATP synthesis in the cardiomyopathy hamster rats and correlation between protein carbonyl content and plasma cardiac troponin (cTnT) was reported (157).

Ischaemic modified albumin (IMA) is an unstable form of human serum albumin (HSA), which is binding site for transition metal ions including cobalt, copper and nickel. Myocardial ischaemia generate number of free radicals, acidosis develops. As myocardial ischaemia could reduce myocardial perfusion, this led to in N-terminal amino acid unstable to binding to copper (Cu^{++}). So, Copper (Cu^{++}) is released from weak binding sites on plasma proteins. Cu^{++} converted to Cu^{+} by ascorbic acid in the circulation. Cu^{+} reacted to oxygen and produce superoxide radicals (O_2^{-}). After that, superoxide dismutase (SOD) dismutase the O_2^{-} forming hydrogen peroxide (H_2O_2) that can degraded by catalase enzyme. The releasing of Cu^{++} ions is scavenged by albumin but tightly bound to the N-terminus. Albumin bounded-copper is damaged by hydroxyl free radicals that remove three N-terminal amino acids and Cu^{++} ions to repeat the mechanism (Figure 10) (158).

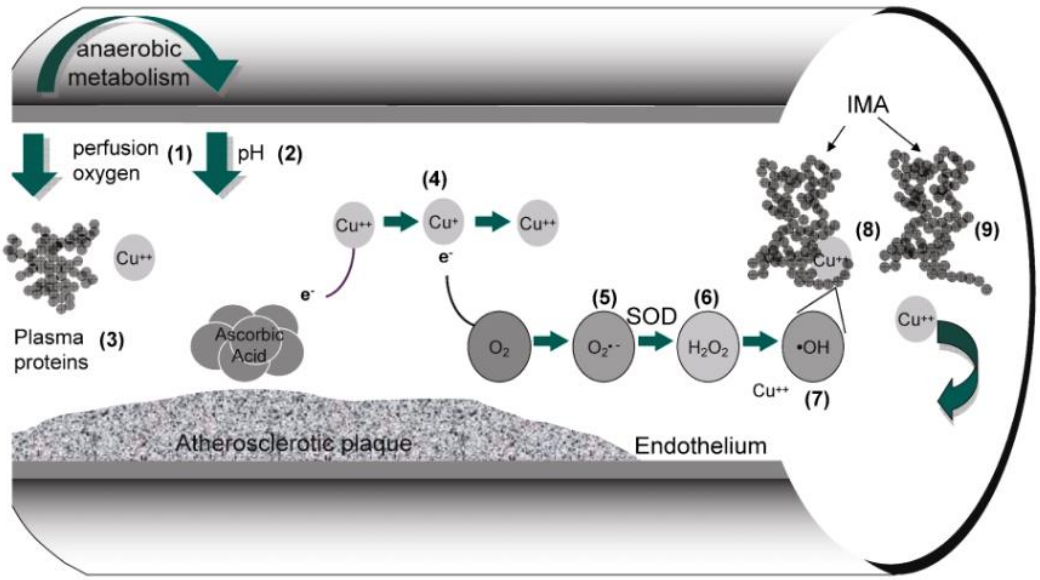


Figure 10 Mechanism of Ischaemic modified albumin generation (158)

The previous report showed the releasing of fatty acid in myocardial ischaemic condition could reduce the ability of albumin to bind to cobalt, by changing conformation of N-terminal region (159), due to conformation change of N-terminal site. Moreover, clinical data showed that plasma IMA level rapid rose in patient after balloon inflation, fall at 6 hours and return to normal level at 24 hours (160). Interestingly, the rise of plasma IMA was occurred earlier than cardiac troponin and natriuretic peptides. Measurement of IMA can be performed by *in vitro* albumin cobalt binding (ACB) assay that measure the alteration of albumin metal binding. The elevation of IMA by ACB assay can be an early indicator of myocardial infarction. The previous study of acute myocardial infarction reported diagnostic sensitivity of cTnI test alone was 23.9% and ACB test alone sensitivity was 39.1% but diagnostic sensitivity was increased to 55.9% when used the combination diagnostic (161). In addition, the diagnostic sensitivity of the combination between cTnI and IMA at 1 to 6 h time point was 86.7% and 6 to 12 h time point was 93.5% (Figure 11).

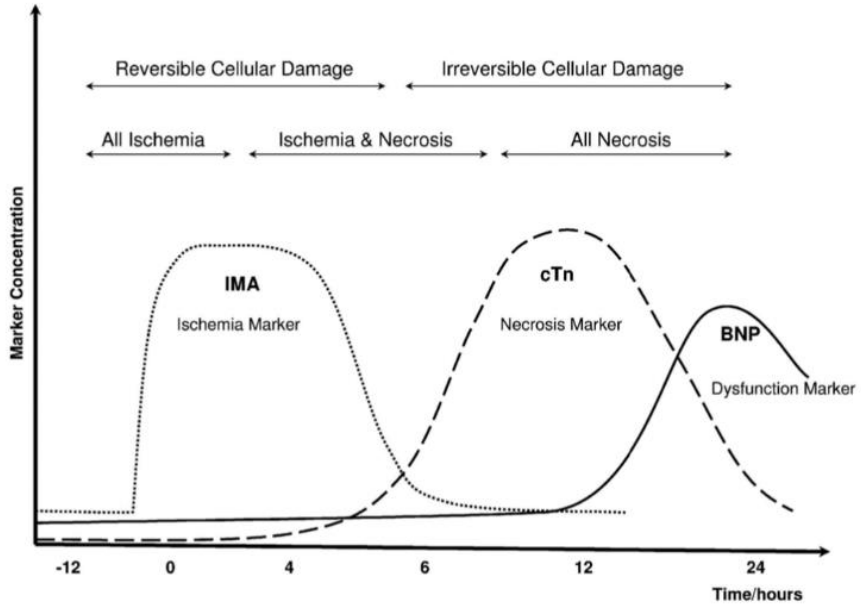


Figure 11 Release kinetics of IMA in relation to standard cardiac biomarkers including cardiac troponin, cTn and natriuretic peptide, BNP (158).

2 Secretory leukocyte protease inhibitors (SLPI)

2.1 Biological and biochemical property and effects of SLPI

The secretory leukocyte protease inhibitor (SLPI) is an 11.7 kDa boomerang-like shape protein non-glycosylate protein (162), containing two whey acidic protein (WAP) domains linked by four disulfide bonds (163) (164). The SLPI is WAP II or C-terminal domain has been known to involve in the protease inhibitory activity (165), which selectively target serine protease enzymes (Figure 12). The protease inhibitory domain resides at residues 67-74 (165), in particular Leu72-Met73- Leu 74 are crucial for antiprotease activity (166). One of the most abundant inflammatory serine proteases is neutrophil elastase (NE). Inhibition of neutrophil elastase (NE) require the association between SLPI and fibronectin or elastin by tissue transglutaminase-2 and plasma factor XIIIa (167). Moreover, SLPI can inhibit cathepsin G, chymase, chymotrypsin, trypsin, and tryptase activity (18, 19, 168, 169), inhibit the production and activity of matrix metalloproteinases (MMPs) (170). The inhibition of serine protease plasmin by SLPI blocks the plasminogen activator through annexin A2 leading to reduced plasmin generation (171).

Although the protease inhibitory activity of SLPI is independent on WAP domain I, the WAP I or N-terminal domain possesses variety of beneficial effects. The WAP I domain is essential for broad-spectrum antimicrobial activity (172-174). SLPI can directly kill microorganisms, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus fumigatus* (172-175). Moreover, SLPI could interfere the human immunodeficiency virus 1 (HIV-1) infection to macrophage by competitively binding to annexin II or scramblase 1 and 4, which are essential binding partner for HIV-1 on T-lymphocytes and macrophages cell membrane (176, 177). In addition, scramblase is a binding partner protein for SLPI involved protein passing through the intracellular (178).

It has been known that SLPI express in various types of cells, including human epithelial cells, immune cells, and fibroblasts (179-181). A high concentration of SLPI was secreted in saliva and mucosal tissue. The concentration of SLPI in the saliva is 30 times higher than in the circulation (182). Human SLPI protein was

encoded from the region of 678 kb on chromosome 20q12-13.2 (164). SLPI mRNA expression is upregulated by several types of pattern recognition receptor ligands, including Toll-like receptors (TLR) ligands (175, 183, 184). Therefore, microorganisms are able to induce SLPI expression from their cell components, including lipopolysaccharide (LPS) of a bacterial cell wall, viral RNA, and parasites (185-188). In addition, SLPI expression in epithelial cells can be stimulated by several types of cytokines including tumor necrotic factor (TNF), interleukin-1 beta (IL-1β), transforming growth factor alpha (TGF-α), insulin-like growth factor 1 (IGF-1), progesterone, and corticosteroids (189-191). In macrophages, Interferon (IFN) and IL-10 have been shown to upregulate SLPI expression in contrary to IL-6 that found to downregulate the expression of SLPI (192, 193). Interestingly, an observation that IFN-γ can reduce TNF-α and nitric oxide (NO) production in macrophages to tolerance to SLPI (183, 194). Although SLPI known to inhibit NE activity, but NE itself could regulate SLPI expression (195). This information suggested that pro-inflammatory cytokines and cell components regulate SLPI expression.

Although SLPI can inhibit the protease activity, but the protease can also inactivate the SLPI itself. Secreted SLPI can be inactivated by myeloperoxidase-catalyzed oxidation, by activated neutrophils (196). Previous study reported that SLPI could be digested by matrix metalloprotease-9 (MMP-9), which subsequently suppress the protease inhibitory activity of SLPI (197). In another way around, cleaved-SLPI loses its ability to inhibit monocyte MMP-9 synthesis, implying that excessive MMP-9 production can overcome SLPI (175, 197). Similar to the case of MMP-9, chymase is also known to cleave and suppress the protease inhibitory activity of SLPI (18, 198). SLPI can also be digested by cathepsins B, L, and S, which renders their anti-neutrophil elastase function inactive (199). This also cause the reduction of half-life in circulation (200).

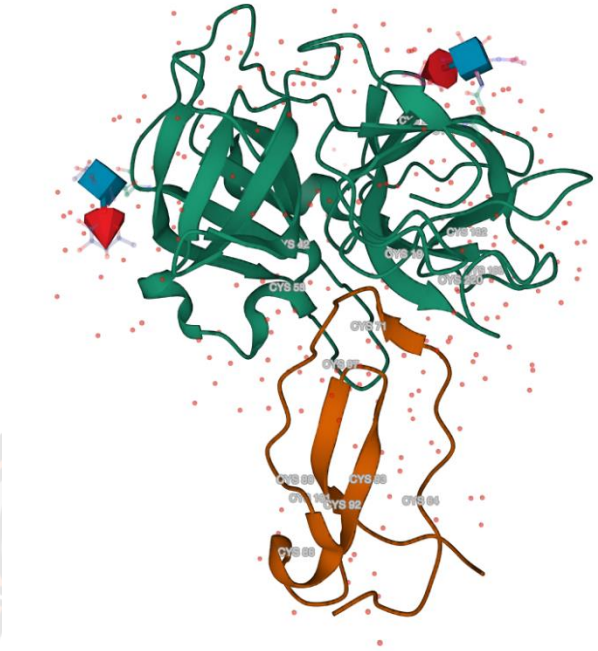


Figure 12 3D structure of SLPI and disulfide bridge (PDB ID: 2Z7F)

2.2 Roles of SLPI in physiology and responses

2.2.1 SLPI in regulating cell proliferation, differentiation, and apoptosis

Roles of SLPI in cell proliferation has been well described in cancer model, in which SLPI promotes cell proliferation by transcriptional activation of the cyclin D1, which important regulator of cell cycle progression and activate cell proliferation (20). In addition to activation of cell renewal, anti-apoptotic activity of SLPI has also been reported. SLPI could inhibit TNF-α-induced apoptosis by downregulating pro-apoptotic caspase-3 (24). The evidence of SLPI in several types of cancer has been reported and also mentioned in the latter section in this review article.

2.2.2 Physiology of SLPI in response to infection and inflammation

The anti-microbial activity of SLPI has been known to be dependent on the N-terminal domain of SLPI (201-203), which related to the cationic nature of the SLPI (204) that allow the protein to adhere to and destabilize bacteria's anionic cell membrane (203, 205). Not only destroying the bacterial cell membrane, SLPI can also inhibit the mRNA translation process by interfering the ribosome to access the Shine-Dalgarno region, which prevents initiation of protein synthesis (206, 207). The anti-proteolytic effect of SLPI may not be necessary for it to serve as an LPS inhibitor.

The antibacterial activity has been studied in several microbial models including *E. coli, Staphylococcus aureus, P. aeruginosa and Staphylococcus epidermidis* (208) *Neisseria gonorrhoeae*, group A *Streptococcus*, *Mycobacterium bovis*, and *Mycobacterium tuberculosis* (209-212). Increasing in serum SLPI levels have been reported in human sepsis and experimental endotoxemia. In sepsis patients, the maximal multiple organ failure ratings and serum SLPI concentrations are highly correlated (213). SLPI-deficient animals experienced severe inflammatory responses with elevated serum IL-6 and led to a considerable rise in morbidity and mortality rates. In the absence of SLPI, macrophages, which are the primary LPS responders, produced more IL-6 and high-mobility group (HMG)-1 protein, a late mediator of endotoxin lethality. (20, 214) These results highlighted that SLPI can provide a protective role in septic shock. In addition, studies in animal model suggested that

SLPI may regulate host susceptibility to bacterial infection. SLPI KO mice subjected to cecal ligation and puncture (CLP) were substantially more vulnerable to endotoxin shock and sepsis (214). Study from Nishimura, *et al.* show that SLPI-null mice showed worsen clinical outcomes of lung infection with the intracellular bacterial pathogen mycobacterium (215).

Anti-inflammatory activity of SLPI has been shown in macrophage responses to bacterial lipopolysaccharides (LPS) (216). The predominant pathway of SLPI for regulating anti-inflammation is due to the attenuation of the degradation of the I-κβ (inhibiting factor of NF-κB) from the ubiquitin-proteosome pathway (217). Following cell activation, I-κβ is quickly destroyed, allowing NF- κB to enter the nucleus and regulate inflammatory gene transcription (20), consequently reduce the level of inflammatory cytokines. On the other hand, SLPI can inhibit the NF-κB signalling pathway by entering the cytoplasm and nucleus to compete with the NF- κB component p65 to associate with the promoter regions of the IL-8, TNF-α (218), IL-10, and IL-1β (219). Numerous LPS-responsive elements, such as NF-κB/rel binding sites, are found in the promoters of LPS-inducible genes, such as NO synthase and TNFα (220).The integrins function as co-stimulators of macrophage in response to LPS, which could be blocked by SLPI. This could subsequently interfere signalling activation for LPS-induced NO and TNF-α production (220).

Epithelial cells, which was recognized the bacteria, will release the B-cell activating factor (BAFF) this is a signal to stimulate B lymphocytes (221). B cell will stimulate the NF-κB to produce the cytokines regulating class switching of antibody such as IgG and IgA. SLPI can attenuate the class switching antibody process by directly eliminating bacteria and inhibiting NF-κB signalling (221).

The fungicidal activity of SLPI has been shown in metabolically active *Candida albicans* and *Aspergillus fumigatus* (222) in dose-dependent manner. Although the antimicrobial activity of SLPI, similar to other cationic antimicrobial polypeptides, is restricted to circumstances of low ionic strength, its fungicidal activity was at concentrations higher than 1 mM (for *C. albicans*) or 2 mM (for *A. fumigatus*) (222).

The anti-viral activity of could be hypothesized by the evidence of lack of oral HIV-1 transmission and the decreased mother-to-child HIV-1 transmission by the

mother's milk, which may be due to the high concentrations of SLPI in salivary (223). Similar to this, low rates of perinatal HIV-1 transmission have been linked to high quantity of SLPI in vaginal secretions (223). The possibly explanation of reducing HIV-1 transmission by SLPI is due to the interference of viral invading process to target cells, and subsequently limit viral replication (177), which independent to SLPI anti-protease activity. Furthermore, it appears that anti-HIV-1 effect of SLPI require a strong binding of SLPI on monocyte surface molecules or receptors (224). The phospholipid (PL) binding protein annexin A2 (AnxA2) and the phospholipid scramblases 1 and 4 (PLSCR1, PLSCR4) are membrane-associated proteins regulate the membrane phospholipids mobility (177). Annexin A2 has been identified as a receptor for CMV (225) and respiratory syncytial virus (226), and later as a molecule that require for HIV-1 infection (227). SLPI was proved to be a binding partner of AnxA2 and disrupt the HIV-1 binding and invading the macrophage. In addition, PLSCR1 and PLSCR4 interact directly with the CD4 receptor at the cell surface of T lymphocytes, where HIV-1 association is required for infection. Since SLPI can bind to the similar region of cytoplasmic domain of PLSCR1, that require for CD4 receptor binding.

2.3 Role of SLPI in non-communicable diseases (NCDs)

Although roles and effects of SLPI has been studied for decade, but the majority of the evidence was highlighted in infectious and immunity, which has been intensively reviewed by several articles. However, the review literature concerning the roles and effects of SLPI in non-communicable diseases (NCDs) is still required. Here, we collect the information from the published articles and summarized in several disease model.

2.3.1 SLPI and neurodegenerative diseases

Secretory leukocyte protease inhibitor (SLPI) is commonly found in mucosal surfaces including saliva, epithelial cells of respiratory tract and urogenital tract (228, 229). However, SLPI was showed the response to central nervous system (CNS) injury that including cerebral ischemia and spinal cord injury (230, 231). The focal

cerebral ischemia model by unilateral middle cerebral artery occlusion (MCAO) showed the upregulation of SLPI in ipsilateral cortex by cerebral ischemia at 12 to 48 hours and the SLPI level continually increased after 5 days of MCAO (230). Furthermore, overexpression of SLPI significantly lower neurological deficit scores. (230). The neuroprotective effect of SLPI also demonstrated in traumatic CNS injury model that performed moderate contusion injury at T11. The SLPI mRNA express at 1 day after the injury whereas SLPI protein could be observed at 3 days after injury. In addition, administration of recombinant SLPI significantly improved motor function that assessed by Basso Mouse Scale (BMS). Moreover, receiving SLPI could increase tissue sparing, myelin integrity, number of ventral horn neuron and density of serotonergic axons caudal to the lesion (231).

2.3.2 SLPI in Respiratory disorders

In respiratory airway, balance of proteases and anti-proteases were indicated to immune response in inflammatory area, especially in cystic fibrosis (CF). CF cause ineffective innate immunity by transmembrane conductance regulator mutation that impair ion channel and mucociliary clearance. These led to chronic pathogen colonization from *Pseudomonas aeruginosa*, and inflammatory response which located in both of upper and lower airways as reservoirs of infection, so discrimination of pathogen located lead to therapeutic treatment for specific immune compartments. The has been reported the level of proteases and anti-proteases concentration were significantly higher in sputum of CF patients compared to nasal lavage (NL) from healthy subject, as 10-fold of neutrophil elastase (NE) and 5000-fold of SLPI. Furthermore, NE/SLPI ratio in NL was 726-fold higher than sputum, respectively (232). Likewise, NL and sputum of CF patients before and after intravenous (IV) antibiotic therapy, IV antibiotic treatment was significantly promoted on inflammatory response in lower airway but trend to decreased in upper airway. MMP9/TIMP1 and NE/SLPI ratio were significantly higher in sputum when compared to NL. Besides, NE/SLPI ratio was 10-fold higher in NL compared to healthy people. Upper airway was indicated delayed anti-proteases and proteases response to IV-antibiotic therapy than lower airway. Then, changes in microbiological

patterns after treated with antibiotic therapy that associated with changes of protease and antiproteases imbalances (233). For the protease activity in animal model, the clinically significant of SLPI has been reported in genetic modified animal model lacking SLPI expression, the bleomycin was used to develop lung fibrosis model in mice, which found that there was an increase in MMP9 activity in *slpi*$^{-/-}$ mice, which related to an impairment of collagen gene expression but reduce lung fibrosis. In addition, the protease mediated TGF-β activity was increased above their increased in wild-type mice after *slpi*$^{-/-}$ mice treated with bleomycin that impair collagen gene expression but minimal reduction of lung fibrosis so SLPI knockout could not prevent the lung fibrosis development following bleomycin-induced lung injury (234).

Not only, CF was the cause of the shift between protease and anti-protease balance, but also found in chronic obstructive pulmonary disease (COPD) following cigarette smoking. The study of differentiated nasal epithelial cells (NECs) and nasal lavage fluid (NLF) of smokers and non-smokers reported an increase in SLPI level in NECs and NLF from smokers. The interferon-sensitive response element (ISRE) binding sites were one of regulatory sites of SLPI promoter (235). Transcription factors of interferon signalling pathway, STAT1 could regulated SLPI expression in epithelial cells and lungs of *stat1*$^{-/-}$ mice compared to wild-type. This finding showed that SLPI regulation and activity in nasal mucosa were induced by smoking (236).

SLPI was downregulated in both of human and mice of severe asthma (SA) in correlation with poor lung function. This could be due to the upregulation of interferon- gamma (IFN-γ) in chronic inflammatory model. The IFN-γ attenuate SLPI expression in airway epithelial cells could indirectly activate protease activity from of mast cell, which contribute to the severity of involved in increased airway hyperresponsiveness (AHR) in mice (237). SLPI seems to have therapeutic benefits since overexpression of SLPI could ameliorate AHR, and more efficiently when treated in combination with corticosteroids (CS). The effective treatment of severe asthma patient was bronchial thermoplasty (BT) which is an endoscopic therapy targets the smooth muscle in the lungs (238). This technique shows to reduce several gene expression that involve in airway inflammation and remodelling in patients with severe asthma. Interestingly, the results showed the SLPI was observed in patients

who had less than 2 exacerbations post-BT. (239) Similarly, Ano S. et al reported that responsive patients showed SLPI gene upregulation, which suggests that SLPI could potentially be useful for identifying risk of exacerbations post-BT (240).

2.3.3 SLPI in Urology Obstetrics and Gynaecology

In the second trimester of pregnancy, short cervical length (CL) was strong predictor of spontaneous preterm delivery (241). The association between short cervical length (CL) and the level of neutrophil elastase (NE), SLPI, and IL-8 has been reported in cervical fluid. Although only cervical fluid NE level, but not SLPI, show correlated to CL (242), but SLPI and progranulin (PGRN) concentration are positively correlated with inflammatory cytokines in cervical mucus samples from 23-26 weeks of gestation reflecting risk of preterm delivery (PD). The finding from the same study in animal model also showed the similar finding about an increasing in SLPI mRNA expression in pregnant mice treated with progesterone supplement. The overexpression of SLPI exhibit anti-inflammation and results in attenuation of cervical remodelling (243). PD is mostly found as a complication in pregnancies after *in vitro* fertilization (IVF). Serum SLPI level can potentially be the best predictive markers for PD, similar to HE4 and IL-13 (244). SLPI was also measured in menstrual fluids and found as one marker among five factors that moderate intraparticipant agreement, but limited variation exists in the selected proteins in menstrual fluid within and between cohort of women (245). Furthermore, the SLPI level in cervical mucus seem to higher in unexplained infertility patients than control group (246). SLPI is also upregulated in endometrial biopsies from persistent mating-induced endometritis (PMIE) and could potentially be a diagnostic marker with 100% sensitivity and 78% specificity (247).

SLPI is crucial for embryos development and implantation. Correlation between *slpi* mRNA expression and embryos development progression were evaluated after the 8-cell stage. The expression of slpi mRNA was lower in *in vitro* than *in vivo* embryos. Correspondingly, *slpi* knockdown by antisense oligonucleotides diminished embryos development speed and implantation rate compared with *slpi* sense-transfected embryos and *in vitro* controls (248).

2.3.4 SLPI in Liver diseases

Acute liver failure (ALF) cause by an exceed of hepatocyte death and innate immune response activation. Acetaminophen-induced ALF (AALF) is mostly found in ALF patient that led to immunoparesis and innate immune response impairment (249). SLPI in hepatic and circulatory concentration were increased in AALF. SLPI also expression in biliary epithelial cell and macrophages in the necrosis area. SLPI was mediated inflammatory response in AALF by modulating monocyte and macrophage function by reducing NF-κB p65, TNF-α, and IL-6 and preserved IL-10 secretion following LPS challenging (250). Besides, SLPI and IL-6, in plasma of donor, were two candidates of risk-prediction biomarker for organ donor and recipient. It has been reported that SLPI can be used as a predictive marker for liver transplantation(251).

2.3.5 SLPI in Kidney diseases

Acute kidney injury (AKI) was common complication after cardiac surgery. Biomarkers play an important role for early diagnosis of AKI patients. It has been found that serum and urinary SLPI mRNA and protein level significantly increased in AKI patients (252). Urinary SLPI level is strongly corelated with SLPI mRNA level in the kidneys, reflecting organ damage associated to SLPI level (253). In kidney transplantation, SLPI concentration in discarded cold-preservative solution can be used as biomarkers for evaluation of the graft quality in kidney transplantation. The level of SLPI is high in delayed graft function or rejected graft post-transplant patient. In addition, SLPI could discriminate estimated glomerular filtration rate (eGFR) in low-risk patients (254). In patients who developed AKI after thoracoabdominal aortic aneurysm repair (TAAA) showed significantly increase in serum SLPI level. Multivariable logistic regression was also showed significantly related between SLPI level at 12 hours after ICU admission and AKI. The sensitivity and specificity of SLPI detection for AKI prediction was 76.47% and 87.5% , respectively, with the optimal cut-off 70.03 ng/ml at 12 hours after surgery (255). The survival rate of kidney transplantation of decreased donors (DD) was lower than living donors (LD)

(256). Transcriptome analysis from kidney biopsies showed SLPI was 203 fold upregulated in DD when compared to LD, which reflected to early donor injury, acute and adaptive immune response that related to inflammation, cell death, remodelling and fibrosis (257). In ischemia/reperfusion (I/R)-induced AKI mice suggest the potential of renoprotection underlying SLPI and SERPINA3M upregulation which related to apoptosis and inflammation (258).

2.3.6 SLPI and ocular diseases

Cellular and tissue homeostasis were maintained by balance between protease and anti-protease. The protease and antiprotease balance alteration was also involved in ocular diseases especially, keratitis, corneal ulceration and endophthalmitis (259, 260). In *S. aureus* keratitis and epithelial defects of murine model showed high level of MMP-8, IL-1, IL-6, TNF-α and SLPI while undetectable SLPI expression in normal control corneal (261). Similarly, Reviglio *et al.* reported that there is no SLPI express in normal ocular tissues but it can be induced by micro-organism invasion. This study showed, SLPI expression protect rat corneal, vitreous and retina tissue from inflammatory cells that release proteolytic enzyme after invaded by *S. Aureus* endophthalmitis (260, 261).

2.3.7 SLPI in bone, cartilage, and dental disorders

The inflammation and metabolic stress can also result in the bone and cartilage disease especially, osteoarthritis (OA) by upregulating the cartilage destruction (262). Cartilage destruction was caused by the upregulating extracellular matrix degradation enzyme and/or downregulating cartilage extracellular matrix molecules from the inflammation (263, 264). Moreover, OA-associate protease was not only alter the protease enzyme activity but it was regulated by cellular inhibitory molecules which is tissue inhibitor of matrix metalloproteinase (TIMPs) (264). Interestingly, treatment with product of inflammation or metabolic stress in chondrocytes including IL-1β, HIF-2α, or ZIP8, showed the upregulation of SLPI in this cell (20, 265). In addition, SLPI is specifically upregulated in OA cartilage and blood serum in both of OA patients and OA mice. These could highlight the application of SLPI as a potential

biomarker of OA (265). Similarly finding also suggests that SLPI is tissue dependent release from intra-articular human OA knee. (266).

Balance between osteoclast and osteoblast regulate osteoporosis. Several factors have been known to involve in bone differentiation and mineralization including, collagen I (Col I), osteocalcin (OCN), and alkaline phosphatase (ALP), dentin saloprotein (DSP), dentin phosphoprotein (DPP), dentin sialophosphoprotein (DSPP), dentin matrix protein- 1 (DMP-1X), and bone sialoprotein (BSP) (267).

Furthermore, pre-treatment osteoblast cells (MC3T3-E1 cells) by recombinant SLPI accelerates the adhesion and migration on Ti surfaces (268), increased cell viability, mineralization, and pre-osteoblasts differentiation. This could be due to SLPI activate upregulation of ALP, DSPP, DMP-1, BSP and Col I (269). Similar findings have been reported in odontoblast-like MDPC-23 cells that pre-treatment with SLPI upregulated gene expression of BSP, OCN, Col I, osteonectin (ON), MMP-2 and MMP-9 but downregulate DSPP.

SLPI show crucial role in odontoblasts and predentin on post-natal (PN) day of mice. SLPI was expressed in odontoblasts and predentin on PN4, under dentin and apical region on PNA10 to PN15 and under layer of odontoblasts and in odontoblasts on PN20. This study hypothesize that SLPI may regulated MMP-2 and -9 in odontoblasts during dentin matrix formation related to differentiation and mineralization (270). In junctional epithelium (JE), Odontogenic ameloblasts-associated protein (ODAM) promotes JE-related gene expression, as well as regulate expression of SLPI (271). SLPI was found as key molecules that involving in periodontal tissue homeostasis during biophysical force (BF)-induced tooth movement (BTM) by alveolar bone AB remodelling. SLPI could induce osteoblastogenic genes including runt-related transcription factor 2 (Runx2), RANKL and MCSF expression combined with compression or tension so SLPI could be target molecule for BTM intervention (272). Furthermore, PDL from healthy patients was showed cytoplasmic SLPI expression that negatively correlated to LPS-induced stimulation of IL-6 and MCP-1 (188).

Severe congenital neutropenia (SCN) was haematological disorder that impaired promyelocutic proliferation and maturation with neutrophil count < 500

cells/μL (273). In dental pulp cells with SCN showed downregulation of ELANE and SLPI expression that consequently cause the attenuation of cell proliferation, attachment, spreading, colony formation, and wound healing with elevated reactive oxygen species (ROS), apoptosis and inflammation (274).

2.3.8 SLPI in Cancer

In cancer, increased SLPI expression has been reported in several types of cancers. Therefore, SLPI can potentially be an ideal biomarker for diagnosis of cancer. Moreover, elevated level of SLPI also found to be involve in metastasis ability of some cancer cells. In this part, the role of SLPI in different types of cancer will be discussed.

There is high SLPI expression in colorectal cancer tissue. Knockdown of SLPI by siRNA could attenuate cancer cell proliferation, migration and invasion that related to downregulation of AKT signalling (162). Immunohistochemistry study showed upregulation of SLPI is associated with pathologic characteristic of colorectal cancer. This suggests that SLPI could be used an indicator of progression and metastasis in colorectal cancer patients (275).

Pancreatic ductal adenocarcinoma (PDAC) also express high level of SLPI, while SLPI gene silencing showed a significant reduction in cancer cell proliferation, increased the apoptosis, and attenuated cell migration and invasion (276). Pancreatic cancer tissue or cell line (Bxpc-3 and Panc-1 cell) showed high SLPI expression level. Similar to other studies, SLPI gene silencing, by shRNA-SLPI, significantly reduced cell viability, suppress cell proliferation and induced cell apoptosis (277).

In gastric cancer**,** evaluation of 68 cases of gastric cancer tissue showed high expression of SLPI, which associate with the survival time, clinical classification, and tumour size. An in vitro experiments reviewed that upregulation of SLPI enhance cancer cell proliferation and metastasis through regulating P53, Bcl-2 and Caspase-8 expression (278).

Bioinformatics analysis and confirmation of gene and protein expression showed downregulation of SLPI in breast cancer (BC) tissues. Moreover, SLPI level was also negatively correlated with estrogen receptor (ER) and progesterone receptor

(PR) but positively correlated with IL-17 receptor B (IL17B) expression (279). High SLPI secretion also correlated with the aggressiveness of triple-negative breast cancer (TNBC) 4T1 cell. SLPI secretion associated with spontaneous lung metastasis from 4T1 tumours orthotopically implants in mice and worse outcome patients. Interestingly, SLPI was found physically interacts with the retinoblastoma tumour suppressor protein (Rb) and FoxM1 form to be Rb-FoxM1 complex. This complex released FoxM1 that may activate breast cancer metastasis targeting gene, FoxM1 (280). Comparison of mouse breast cancer cell line 4T1 and its highly metastatic 4T1.2 clone showed that SLPI was a dominant secreted protein that highly expression in both of medium and cell lysates of 4T1.2 cells (281). Pre-treatment of SLPI in murine (F3II) and human (MCF-7) breast tumour cells decreased E-cadherin expression and re-localized to cytoplasm and β-catenin re-localized to the nucleus. Similarly, stable F3II overexpressing SLPI showed disrupted E-cadherin and β-catenin complex that related to increasing of Bax/Bcl-2 ratio and p21 protein level, decreasing of c-Myc protein and Cyclin D1 and Claudin-1 levels (282). This suggests negative effect of SLPI in breast cancer.

Cancer metastasis required intravasate into the lymphatic system or vasculature and extravasate (283). SERPINE2 and SLPI was secreted from polyclonal mouse model which were necessary and sufficient for vascular mimicry and ensure their perfusion by anticoagulant effect. Lung-metastasis relapse patient showed overexpression of SERPINE2 and SLPI, suggesting that these two molecules can promote metastatic progression in human cancer (284). In addition, previous study showed that serum SLPI and SERPRINE2 level was higher in Papillary thyroid cancer (PTC) patient compared to multinodular nontoxic goiter (MNG) and control group (CG) (285). Likewise, results from DNA microarray indicated SLPI could be a potential biomarker for PTC (286).

There was a report showed high SLPI expression in head and neck squamous cell carcinoma (HNSCC) without human papillomavirus (HPV) driven in tumour tissue of smokers higher than non-smokers similar to increasing annexin A2 gene expression level. (287) It has been report that high SLPI expression level was associated with higher risk of HSCC with smoker history (288). Interestingly in oral

squamous cell cancer (OSCC) tissue, only SLPI expression has been found in association with lymph node metastasis, overall survival (OS) and disease-specific survival (DSS) (289). Additionally, SLPI was one of fourteen biomarkers protein candidate from saliva samples of OSCC that showed higher expression. In oropharyngeal cancer (OPC), although the oral gargle SLPI levels was not significantly associated with OPC, but it was associated with tonsillectomy and HPV. Besides, increasing SLPI level was also related to increasing odds of incomplete treatment response (290). SLPI play role in invasion in human gingival carcinoma Ca9-22 cell line (291). SLPI-deleted human gingival carcinoma Ca9-22 (ΔSLPI) cells showed small lamellipodia/dorsal ruffles slower moving in ΔSLPI cells that associated with well-developed intermediate filament bundles in desmosome junction compared to wtCa9-22 cells (292), and exhibit lower migration (293). These studies suggest that SLPI involve in cancer cell migration and invasion.

In contrary to the above information, SLPI downregulation has been reported to correlated with cancer progression. Brushed biopsy samples indicated that SLPI progressive declining trend between healthy normal and oral premalignant lesion tissue (OPMLs) tissue and further decreasing in OSCC lesion tissues. This indicated that reduction of SLPI is correlated with oral cancer progression (294). Similar to oral leukoplakia (OL), which is the most common oral precancerous lesions and be a potential predictive tool for the malignant transformation, immunohistochemical and histological grades is negatively correlated with the SLPI level. An *in vitro* biological effects of SLPI in Human Premalignant Oral Leukoplakia Cell Line (MSK-Leuk 1) and Wayne State University-Head and Neck 4 (WSU-HN4) cell lines was measured showed that SLPI inhibited TNF receptor-associated factor 1 (TRAF1) regulated cell apoptosis (295).

SLPI involved in bladder cancer (BCa) and prostate cancer (PCa) metastasis that altered by androgen response elements (AREs) located at different position in the miR525 precursor promoter. AREs induce vasculogenic mimicry (VM) formation either decrease PCa and increased BCa metastasis through miRNA525p that altered SLPI mRNA expression (296). AR also promote castration-resistant prostate cancer (CRPC) survival and growth, which correlate to higher serum level of SLPI.

Therefore, SLPI promote CRPC cell survival and growth after androgen withdrawal *in vivo* and *in vitro* (297).

The study in gastric cancer (AGS) cell line showed the high level of SLPI, and lipocalin-2 (LCN2). The LCN2 binding with SLPI is mediated by IL-17. Treatment with IL-17 significantly suppress AGS cell proliferation, clone formation, migration, invasion, cell cycle and cell apoptosis by targeting SLPI (298).

2.3.9 SLPI in Metabolic diseases

Metabolic diseases, which is a cluster of conditions that increase risk of heart disease, stroke, and type 2 diabetes, become a major public health issue. Although several studies have been reviewed on pathophysiology of the diseases, but roles and effects of SLPI in metabolic diseases seems not well-known.

The methyl-CpG-binding protein MeCP2 has been identified as proteins that selectively bind methylated DNA (299). The abnormalities methyl-CpG binding protein 2 (Mecp2) can be found in metabolic disorders (300). Obese human and mice showed upregulation of Mecp2 in white adipose tissue (WAT) that consequently bind to SLPI promotor and regulate SLPI expression. (301). In pathway of fatty acid metabolism regulation and lipid accumulation, Fatty acid transport protein 1 (FATP1) has been identified to be involved in lipid metabolic pathway (273). Study in model of FATP overexpression/interference by differentially expressed genes (DEGs) showed that SLPI is a candidate gene controlling fat deposition and fatty acid metabolism (302).

2.3.10 SLPI in Cardiovascular diseases

Cardiovascular diseases (CVDs) are considered as the major cause of global mortality and disability. The pathophysiology as well as candidate to treatment has been intensively studied, however, there are limit number of studies on the roles and effects of SLPI in cardiovascular diseases, particularly ischemic heart disease (IHD).

Protease enzymes cause widespread destruction and have relatively long half-lives in tissue (303). Moreover, an increasing in the activity of proteolytic enzymes such as chymase, matrix metalloproteinases, calpains, cathepsins, and caspases

contribute to the process of cell death, injury (12). Therefore, inhibition of protease activity can therefore be considered as powerful strategies for prevention of ischaemia/reperfusion (I/R) induced tissue injury. The level of serum neutrophil serine proteases (NSPs) inhibitors from coronary artery disease (CAD) and coronary artery ectasia (CAE) patients were significantly increased, particularly α1-protease inhibitor (PI) and α2-macroglobin (MG). Whereas SLPI and elafin were not significantly increased in CAE and CAD patient that due to vessel could not a main distribution site for SLPI and elafin (304). In addition, atrial fibrillation (AF) incident and circulating SLPI level is positively correlated in diverse population that related with the circulating level of MMP-2, TIMP-2 and VCAM-1 (305). Several studies in *in vitro* model of myocardial ischaemia reperfusion (I/R) injury, by simulated I/R (sI/R) condition, showed that pretreatment of recombinant human SLPI could reduce cardiac myoblast (H9c2) cell death and attenuate activation of p38 MAPK (23). Similarly, the overexpression of SLPI gene could also reduce cell death and cell injury (23), as well as protect the heart from an in vivo I/R injury (306). This could be repetitively showed in isolated adult rat ventricular myocytes (AVRMs) (22) and isolated adult rat cardiac fibroblasts (ARCFs) (307) (Figure 13). The mechanisms are believed to attenuate reactive oxygen species production and apoptosis pathway. The similar findings on cardioprotection effect of SLPI can also be demonstrated in an *ex vivo* (22) and *in vivo* model (308) of I/R injury. Pre-treatment of SLPI or giving during LAD ligation and onset at reperfusion also showed cardioprotective effect against I/R injury. The reduction of infarct size, improvement of cardiac function, reduce intracellular ROS production such as protein carbonyl (PC) and ischaemic modified albumin (IMA), inflammatory cytokines and regulatory apoptotic protein are also intensively studied (308). Interestingly, although the beneficial effects of SLPI is believed to be due to the anti-protease activity, few studies showed that those effect of SLPI might be independent on its anti-protease activity. The study of anti-protease deficiency activity of SLPI as mutant SLPI (L72K, M73G, L74G) can also provide cardioprotection against myocardial I/R injury (309). These data suggested that SLPI has cardioprotective effect in cardiovascular diseases especially, myocardial I/R injury.

Not only for the heart, but SLPI can also provide vasculo-protection on vascular endothelial cell (310) against an in vitro simulated ischemia/reperfusion (sI/R) injury. The similar mechanistic findings have been reported by reducing cell death, intracellular ROS production, attenuation of apoptotic pathway and enhance cell survival pathway. SLPI that derived from vascular endothelial cell could be a angiocine that reduced cardiac cell death(311). Therefore, application of SLPI as a cardio-vasculo protective agent could possible. Previous study from Schneeberger et al, in 2008 by adding recombinant protein of SLPI in preservative solution could restored myocardial contraction in transplanted heart (17). Similarly, isolated thoracic aorta and abdominal aorta ring preserved in normal saline solution supplemented with recombinant protein of SLPI could reduce vessel graft inflammation and tissue degeneration (312). These studies suggest a cardioprotective effect of SLPI and could potentially be a candidate of new drugs.

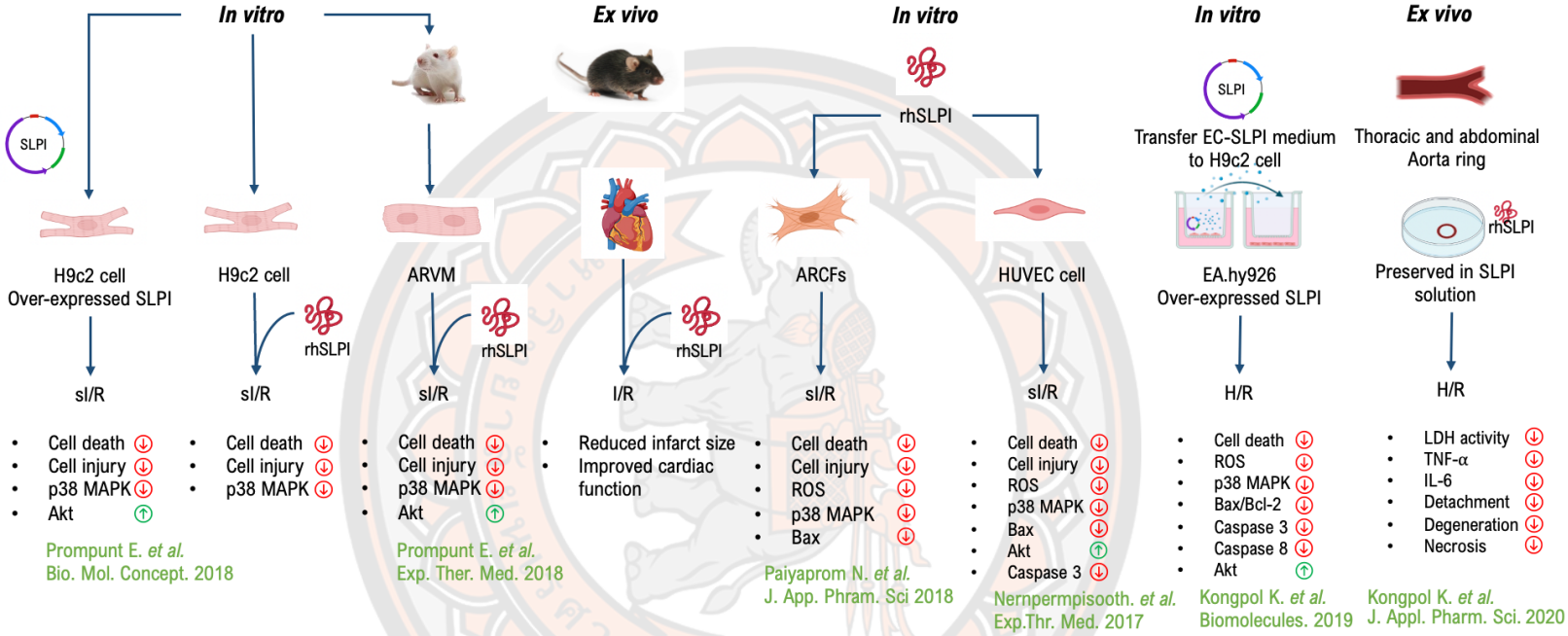


Figure 13 Schematic representation the previous study in SLPI

CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology of this research including material and method. The detail of each topic is described below.

Research Methodology

The conceptual idea of experiment is determining the cardiac physiology and biochemical alteration in Wistar rat model subjected to ischaemia/reperfusion injury. In this PhD dissertation, the study was divided into 2 major parts according to the aim of study, including;

Aim 1 To investigate the effect of recombinant human SLPI on physiological and biochemical function, in rats subjected to *in vivo* ischaemia/reperfusion (Figure 14)

Aim 2. To determine the anti-protease involvement in cardioprotective mechanisms of recombinant human SLPI against ischaemia/reperfusion (Figure 15)

The recombinant human SLPI (rhSLPI) (for aim 1) or anti-protease deficiency mutant of SLPI (for amin 1 and 2) were administrated via intrajugular vein. Determination of cardiac physiology and function were measured by end-systolic pressure (ESP), end-diastolic pressure (EDP), dP/dt max, contractility index (CI), dP/dt min, Tau/e, develop pressure (devP) and heart rate (HR). The sensitivity to infarction and area at risk were measured by tissue staining. Moreover, biochemical alterations including MAPK phosphorylation and signalling protein involve in apoptosis such as p38, Akt, ERK1/2, Bax, caspase 3 and caspase 8 were determined by Western blot analysis as in Figure 14 and Figure 15.

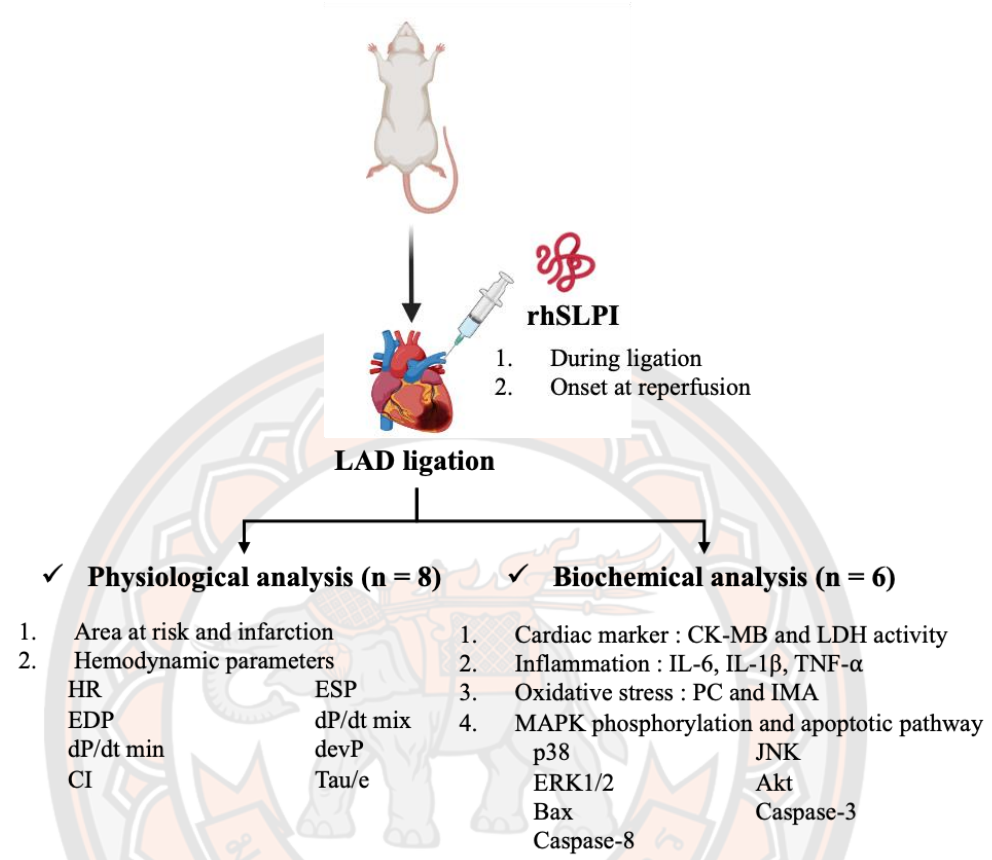


Figure 14 Schematic representation the research methodology of post-ischaemic treatment of SLPI under I/R injury condition

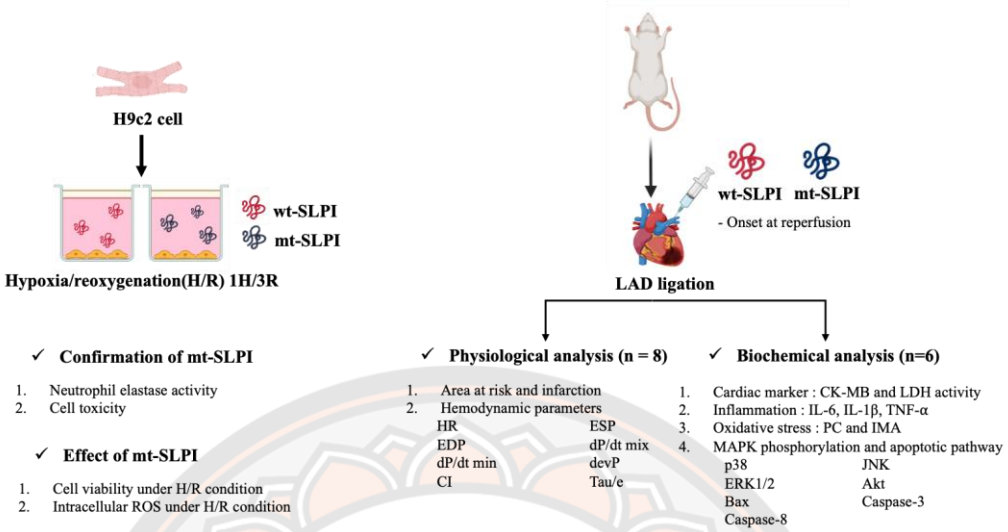


Figure 15 Schematic representation the research methodology of anti-protease deficiency SLPI treatment under I/R injury

Experimental Animals

Adult male Wistar rats weighting 250-300g were purchased from the Nomura Siam International, Thailand. All animals were maintained under environmentally controlled conditions (22 ± 1 °C, 12 h light: dark cycle) Chulalongkorn University Laboratory Animal Centre (CULAC). All protocols used in this study were approved by the Animal Use and Care Committee at Naresuan University (NU-AE620615) and the Institutional Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University, Thailand (Protocol No.1973025). The overall number of animals was used in this study is shown in Table 1.

Table 1 The number of animals in this study

Experiment aim	Number of animals
1	70
2	70
Total	140

1. The effect of post-ischaemic treatment of recombinant human SLPI treatment on rat hearts subjected to an *in vivo* ischaemia/reperfusion.

To determine the effect of post-ischaemic treatment of recombinant human SLPI treatment on cardiac physiology, functions, and sensitivity to infarction, and intracellular biochemical alterations in rat hearts subjected to an *in vivo* ischaemia/reperfusion Recombinant human SLPI (rhSLPI, Sino Biology Inc., Beijing, China) was diluted in sterile phosphate buffered saline (PBS) and rat was injected through intravenous route with 50 µg of SLPI in 500 µl total solution. The dosage of rhSLPI in this study based on the previous reports in an *in vivo* model (313, 314)

1.1 Experimental group assignment

Schematic diagram of experimental protocol, as shown in . Seventy adults male Wistar rats were used in this study, which were divided into 2 experimental groups. All of animals were performed following experiment protocol that including 1. Baseline 30 mins

2. Ligation 30 mins

3. Reperfusion 120 mins

Both of experiments were including 4 animal group which is sham, vehicle, rhSLPI treated at during ligation (rhSLPI-DI) and rhSLPI treated at onset of reperfusion (rhSLPI-OR). Both of treatment group, rhSLPI-DI and rhSLPI-OR, rats were administrated 50 µg rhSLPI at 15 mins of ligation and beginning of reperfusion, respectively. The experiments were including physiological study, forty rats were used (Sham =8, Vehicle = 8, Treatment group =8 x2, 8 rats as spare animals) and biochemical analysis, thirty rat were used (Sham =6, Vehicle = 6, Treatment group =6 x2, 6 rat as spare animals) (Figure 16).

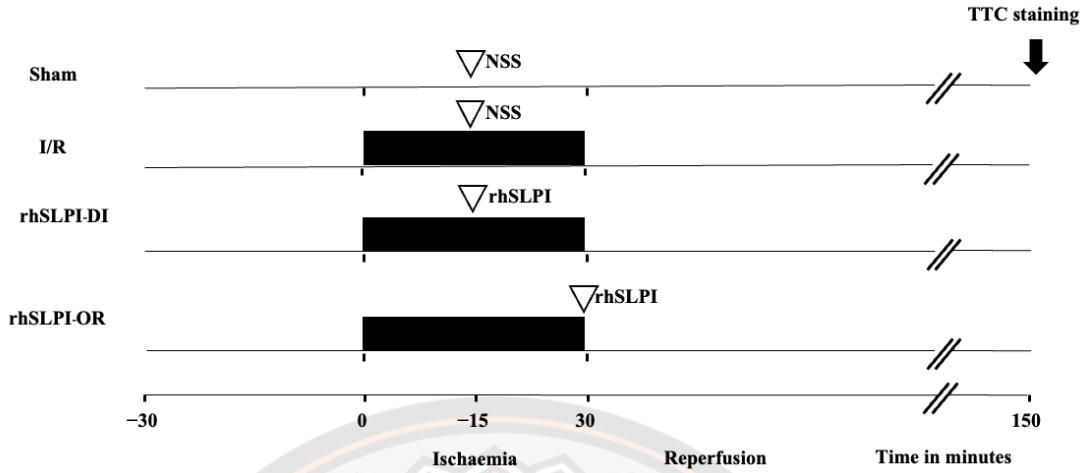


Figure 16 Schematic diagram of experimental protocol

1.2 Surgical preparation of myocardial I/R model in rats

Rats were anesthetized in isoflurane induction chamber followed by tracheotomy for intubation and connected to the rodent ventilator (VentElite, Harvard apparatus, MA, USA). The tidal volume was set at 2.5 mL with a rate of 80 breaths/min. A level of anaesthesia was maintained with isoflurane 1.8-2.0% in 100% oxygen. The body temperature was maintained at 37 ºC using warm water heating pump. Lead II electrocardiogram (ECG) was attached to the rat and connected to the ECG amplifier. The carotid artery was cannulated with Mikro-Tip® pressure catheter for rat(Millar, Houston, Texas, USA) for measuring left ventricular pressure (LVP) parameters. The jugular vein was also cannulated fluid-filled catheter for blood collection and rhSLPI administration. Then, left thoracotomy was performed to reveal left coronary artery. A 6-0 synthetic, monofilament, nonabsorbable polypropylene suture was tied around the left anterior descending (LAD) of the left main coronary artery with the PE-50 tube to simulate ischaemic condition. Regional ischaemia was confirmed with an elevation of ST-segment in lead II tracing and faded whitish colour tissue by visual inspection. The ligation was released after 30 min of ischaemia, and the ligation was released by pulling the tube out of the ligation while the suture was remained at the same place. The reperfusion was continued for 120 min. The ECG and LVP were recorded throughout the experiment with an IOX system (IOX 2.10.8.6, EMKA Technologies, Paris, France) and were stored on a hard drive for later analysis with ECG Auto software (ECG Auto 3.5.5.12, EMKA Technologies, Paris, France). The LVP was analysed for end-diastolic pressure (EDP), end-systolic pressure (ESP), develop pressure (devP), and heart rate (HR). The contractility was inferred from the maximum rate of rise of the LVP (dP/dtmax) and contractility index (CI) defined as the dP/dtmax over the pressure at that point. In addition, the lusitropic indices were determined by the maximum rate of fall of the LVP (dP/dtmin) and Tau. At the end of the protocol, the hearts were quickly excised for infarct size assessment.

1.3 Determination of hemodynamic parameters by Mikro-Tip® pressure catheter for rat (Millar, Houston, TX, USA)

After the animal preparation, the pressure-conductance catheter (Millar, Houston, TX, USA) was advanced to record the left ventricular pressure in left ventricle. All hemodynamic parameters were recorded including end-systolic pressure (ESP), end-diastolic pressure (EDP), dP/dt max, contractility index (CI), dP/dt min, Tau/e, develop pressure (devP) and heart rate (HR).

1.4 Determination of the area at risk and infarct size

After the end of the reperfusion protocol, the LAD was re-occluded and 2% (w/v) Evans blue solution (in PBS) was intravenously injected in order to differentiate the area at risk (non-perfused during the coronary occlusion) from the non-ischemic area. At the end of the surgical protocol, the heart was excised, weighed and transversally sliced in 1-mm-thick slices that were subsequently incubated in 1% (w/v) 2,3,5-triphenyl tetrazolium chloride (TTC) at 37 ◦C for 10 min to define the necrotic myocardium after enhancing the contrast between stained and unstained TTC tissues with a 10% (v/v) formalin (15−20 h) final incubation. The infarct area (TTC-negative) and the non-ischemic area (Evans blue-stained area) were measured by using ImageJ software.

1.5 Determination of Serum Creatine Kinase (MB Isoenzyme) and Lactate Dehydrogenase (LDH) Activity

Serum was collected at the end of the study protocol for determining the level of cardiac biomarkers. The creatine kinase (MB isoenzyme) and lactate dehydrogenase (LDH) activity was analysed by using an automated biochemistry analyser (Cobas c 111 analysers, Roche, Basel, Switzerland).

1.6 Heart tissue homogenization and protein collection

Another set of experiment was performed on another group of rats to assess the biochemical parameters. At the end of the I/R protocol, the heart was rapidly excised, and then the whole ventricular tissue was collected, quickly frozen in liquid nitrogen, and stored at -80°C until analysis. One hundred milligrams of tissue

from each organ were isolated and then homogenised by hand homogeniser on ice. One hundred milligram of heart tissue was homogenized with 1 ml of homogenisation buffer (20 mM Tris HCl pH6.8, 1mM Na_3VO_4, 5 mM NaF and protease inhibitor tables (complete®). Tissue homogenate (100 mg/ml tissue extract) was centrifuged at 14,000 rpm for 10 min at 4ºC. The supernatants were collected and frozen for further analysis

1.7 Determination of protein concentration by Bradford assay

Determination of protein concentration was performed by using Bradford reagent (HIMEDIA®). Five microliter of protein extractions were added into 250 µl of Bradford reagent and mixed by pipetting. The reactions were incubated at room temperature for at least 5 min and measured the absorbance at λ595 nm by spectrophotometer. The protein concentration was determined by using bovine serum albumin (BSA) as a standard protein for calculating and normalizing the amount of total protein that used in ELISA method and Western blot analysis at 100 µg and 25 µg, respectively

1.8 Determination of inflammatory cytokines level by Enzyme-linked immunosorbent assay (ELISA)

The ELISA (ABTS ELISA Buffer Kit, Peprotech®) were prepared at room temperature by gentle mixing. Firstly, the 96-well plate was pre-coated by adding 100 µl of 1 µg/ml capture antibody to each well and incubated overnight at room temperature. Next day, the plated was inverted for removing the liquid and blot on the paper towel. After that, the plate was washed with 200 µl washing buffer solution for 4 times. The blocking solution was added into the well for 200 µl and incubated for 1 h. The reactions were washed for 4 times again. One hundred microgram of tissue homogenates were added into the wells and incubated at room temperature for at least 2 h. The plate was washed again and added 100 µl detection antibody and incubated at the room temperature for 2 h. The plate was washed again and 100 µl of Avidin-HRP conjugated 1:2000 was added and incubated at room temperature for 30 min. Then, the plate was washed and 100 µl of ABTS liquid substrate was added into the well and incubate at the room temperature for colour

development. The sample absorbance was measured by spectrophotometer at 405 nm with correction wavelength correction set at 650 nm.

1.9 Spectrophotometric determination of protein carbonyl (PC) content level

Oxidative induction is a key factor leading to cardiac cell death in myocardial ischaemia and ischaemia/reperfusion injury (315), and subsequently resulted in biochemical modifications of some biomolecules such as protein, lipid, and DNA (316). Ischaemia modified albumin (IMA) and Protein Carbonyl (PC) is proteins that are considered as a modified product on the similar basis of oxidative stress induced protein modification and may have diagnostic potential in acute myocardial infarction (316). In this study, the oxidatively modified proteins including IMA and PC were determined in both serum and cardiac tissue homogenate.

Both serum and cardiac tissue protein carbonyl (PC) content level were spectrophotometrically determined by colorimetric DNPH assay, as previously described [24]. Firstly, protein extracts were diluted to 1:10 with phosphate-buffered saline (PBS). Two hundred microliters of diluted protein extractions were added into 800 µl of 10 mM DNPH in 2.5 M HCl. One millilitre of 20% (w/v) trichloroacetic acid (TCA) was then added and centrifuged at 10,000×g for 10 min at 4 ºC to precipitate protein. The protein pellet was washed 3 times with 1 ml of 1:1 (v/v) ethanol: ethyl acetate and centrifuged at 10,000 × g for 10 min at 4 °C. After final washing, the protein pellet was resuspended in 500 µl of 6 M guanidine hydrochloride and centrifuged at 10,000 × g for 10 min at 4 ºC. The supernatant was collected and measured the absorbance at 370 nm using 6 M guanidine hydrochloride as a blank. The PC content (nmol/mg) was calculated following the procedure used in a previous study (317).

1.10 Spectrophotometric determination of Ischaemic modified albumin (IMA) level

Determination of ischaemic modified albumin was performed by using albumin cobalt binding (ACB) assay, as previously described (317). Two hundred microlitre of serum or cardiac tissue homogenate was added into 50 µl of 0.1% (w/v) Cobalt chloride and mixed gently. The samples were incubated at room temperature for 10 min for adequate cobalt-albumin binding. Then, fifty microlitre of 1.5 mg/ml dithiothreitol (DTT) was added to colorizing the reaction and incubated at room temperature for 2 min. One millilitre of 0.9% (w/v) Sodium chloride (NaCl) to quench the reaction and absorbance measured at 470 nm. The determination of ischaemic modified albumin was calculated by serum-cobalt blank without DTT as a blank of individual sample.

1.11 Determination of MAPKs phosphorylation and signalling protein involve in apoptosis

Principle of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis is method for separating protein sample depending on their migration in electrical field. The migration of protein in electrical field is depend on their charge, size, shape and isoelectric point. Normally, protein is formed to be tertiary structure, which has net electrical charge and pH. The protein should be denatured and neutralized by detergent such as sodium dodecyl sulphate (SDS). Then, the neutralized protein was separated by polyacrylamide gel, which depending on only molecular weight. Sodium dodecyl sulphate (SDS), lauryl sulphate, is an anionic detergent, which has a negative charge in a wide pH range when its dissolve in solvent. SDS bind to protein with 1.4g SDS per gram of protein ration for destroying the complex structure of protein. SDS convert the net charge of protein to negative charge in proportion to its length. Polyacrylamide (PAM) is a polymer from polyacrylamide subunit, which usually used in separate biological macromolecules such as protein and nucleic acid. Interestingly, SDS does not interact with proteins that leading to the separation of protein due to the difference of molecular weight.

Western blot analysis is an important technique to detection and analysis of protein. This technique is based on antigen-antibody interaction by specific binding to protein. After protein was separated by SDS-PAGE, the protein should be transfer onto polyvinylidene fluoride developed negative films. Band intensity in difference lane should be compared on relative expression of protein (308).

The heart homogenate was mixed with equal volume of 2X SDS-PAGE sample buffers, containing 10% (v/v) 2-mercaptoethanol and bromophenol blue dye. The sample was boiled for 10 min. A measured 25 µg of protein was loaded into SDS-PAGE polyacrylamide gel electrophoresis gel for separation. Electrophoresis was performed at 120V for 2 h. After separation, proteins were transferred to Immobilon-P membranes (Millipore) using a semi-dry apparatus under an electrical current of 15 mV, for 45 min. Then, membranes were incubated in a 5% (w/v) of dried skimmed milk powder in TBST solution for 1 h with gentle shaking at room temperature. The membranes were incubated with primary antibodies for phosphorylated-Akt (Santa Cruz Biotechnology, Inc., #sc-7985), total-Akt (Santa Cruz Biotechnology, Inc., #sc-8312), phosphorylated p38 (Cell signalling Technology, #9211), total-p38 (Cell signalling Technology, #8690), Bax (Santa Cruz Biotechnology, Inc., #sc-493), Bcl-2 (Santa Cruz Biotechnology, Inc., #sc-492) caspase 3 (Santa Cruz Biotechnology, Inc., #sc-7148) and caspase-8 (Cell signalling Technology, #9476) (All of primary antibodies were diluted at 1:1,000 in 1% (w/v) skimmed milk + TBST buffer) and the horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit (Merck, #AP132P) and rabbit anti-goat (Merck, #AP106P), which was diluted at 1:5,000 in 1% (w/v) skimmed milk + TBST, after the heart protein was separated in SDS-PAGE. The band intensity quantitation was captured and analysed by ChemiDoc XRS+, Image LabTM 2.0 Software (Bio-Rad Laboratories, Inc.) (318).

2. The effect of antiprotease-deficiency of SLPI on ischaemia/reperfusion injury.

From our previous studies as well as the first part in this dissertation demonstrated the cardioprotection of Secretory Leukocyte Protease Inhibitor (SLPI) against myocardial ischaemia/reperfusion (I/R) injury. Although the beneficial effects of SLPI are believed to be due to the anti-protease activity, few studies show an independent on anti-protease activity of SLPI (319). Furthermore, there are no such evidence confirm that the cardioprotective effects seen from the previous studies is really due to inhibition of the proteases. To answer this research question, the recombinant protein of anti-protease deficient mutant SLPI (L72K, M73G, L74G), which is lack of anti-protease activity, was used to elucidate the cardioprotection in both in vitro of hypoxia/reoxygenation (H/R) and in vivo model of myocardial I/R injury.

2.1 Recombinant protein of anti-protease deficient mutant SLPI

2.1.1 Production of recombinant mutant forms of SLPI (mt-SLPI)

The mutant SLPI plasmid harbouring the substitutions of amino acids that critical for SLPI's antiprotease activity $Leu^{72}Lys$, $Met^{73}Gly$, $Leu^{74}Gly$, (L72K, M73G, L74G) was kindly provided by Professor Joanna Cichy, Jagiellonian University, Kraków, Poland. The cDNA encoding mutant SLPI was generated by polymerase chain reaction (PCR), using RNA isolated from human alveolar epithelial cells as a template, and the mutations was generated by site directed mutagenesis. The cDNA containing mutant SLPI from PCR reaction was inserted to pPIC9 expression vector (Life Technologies, Carlsbad, CA, USA) with the addition of a hexahistidine tag and an enterokinase cleavage site to its N terminus (319). The mutant SLPI-pPIC9 was transformed into *Escherichia coli* TOP10. The transformants were screened using colony PCR and corrected plasmid was transformed into *Pichia pastoris* KM71H. Protein expression level was optimized according to manufacturer's instruction (Life Technologies, Carlsbad, CA, USA).

2.1.2 Purification of anti-protease deficient mutant SLPI

A single colony of mt-SLPI was inoculated in Yeast Extract-Peptone-Dextrose (YPD) broth supplemented with 100 µg/mL zeocin and incubated at 30 ˚C, 250 rpm for overnight. Then, the cell was harvested, re-suspended with Buffered Glycerol Complex Medium (BMGY) broth in a ratio of 1: 10 (v/v) and incubated at 30 ˚C, 250 rpm. After 16-18 h incubation, the cell was harvested and re-suspended to an OD_{600} of 10 in Buffered Methanol-complex Medium (BMMY) and further incubated at 30 ˚C, 250 rpm. The final concentration of 1% (v/v) methanol was daily added. After 7 days incubation, the supernatant was collected and loaded onto nickel–nitrilotriacetic acid matrix (Ni^{2+}-NTA) affinity chromatography (GE Healthcare, Uppsala, Sweden). The recombinant protein was eluted with stepwise gradient of 50, 100, 150, 250 and 500 mM of imidazole in 50 mM Tris-HCL, pH 8.0 and 300 mM NaCl. The purified protein was confirmed by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using an anti-His tag antibody.

2.2 Assessment of inhibitory activities of anti-protease deficient mutant by neutrophil elastase activity

The activity of neutrophil elastase (NE) was measured by using neutrophil elastase activity kit was purchased from Abcam (ab204730, Cambridge, UK). The measurement is based on the ability of neutrophil elastase to proteolytically cleave a synthetic substrate in order to release a fluorophore. The neutrophil elastase was preincubated for 10 min with recombinant human SLPI (wild type; wt-SLPI) and anti-protease deficient mutants (mt-SLPI) at 2:1 [SLPI:NE] molar ratio, at 20°C in 0.1 M Tris-HCl (pH 8.0) with 0.5 M NaCl for 1 h. The SLPI-NE mixture and mutant SLPI-NE mixture were added into reaction mix that containing the neutrophil elastase buffer and substrate, while standard NE was used as control. A reaction was incubated at 37 °C for 20 min. Then, the activity was measured with fluorescence microplate reader (EnSpire® Multimode Plate Reader, PerkinElmer, MA, USA) at 380/500 nm (excitation/emission). The activity of neutrophil elastase was expressed as % relative fluorescence units (RFU) of standard NE control and subtract with sample background.

2.3 Cell culture

The rat embryonic cardiomyocyte cell line (H9c2) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) as ATCC-CRL1446. Cells were cultured in completed Dulbecco's Modified Eagle Medium (DMEM) supplement with 10% foetal bovine serum and 5000 units/mL of penicillin/streptomycin that were purchased from Gibco (Gibco BRL, Life Technologies, Inc., NY, USA). Cells were maintained in humidified atmosphere 95% air and 5% carbon dioxide at 37 °C.

2.4 An *in vitro* Hypoxia/Reoxygenation induce cardiac cell death

An *in vitro* Hypoxia/Reoxygenation (H/R) protocol was modified from previous study (320). H9c2 cells were seeded into 96 well plated at density of 1.0×10^4 cells/well and left until cell density reached 70–80% confluence under humidified CO_2 incubator. Then, the medium was removed and replaced with 100 µl DMEM without serum and overlayed medium with 200 µL Liquid paraffin. Cells were incubated at 37 °C in a humidified CO_2 incubator for 1 h, then replaced with completed DMEM and incubated for 3 h (reperfusion). After reperfusion, cell viability was determined by MTT assay. The optimized timing was considered as timing that caused 50% cell death, which was used throughout an *in vitro* study.

2.5 Determination of cell viability by MTT assay

MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Thermo Fisher scientific. The medium was removed and replaced with 0.5 mg/mL MTT reagent. Cells were incubated with MTT reagent for 4 h in humidified CO_2 incubator. After incubation, the dimethyl sulfoxide (DMSO) was added to solubilize the form-azan crystal. Cell viability was determined using a spectrophotometer at a wavelength of 490 nm, using DMSO as a blank (308).

2.6 Determination of Intracellular reactive oxygen species (ROS) Production

The determination of intracellular reactive oxygen species (ROS) production was followed by the previous report (22) that using 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Sigma, St. Louis, MO, USA). Briefly, cells were cultured in 96 well plates with DMEM. The culture medium was removed and replaced with 2 μM DCFH-DA in DMEM without serum in dark room for 24 h at 37 °C. Then, medium was removed and replaced with DMEM without serum and covered with liquid paraffin, which followed by H/R protocol. After incubation, medium was removed and replace with completed DMEM with DCFH-DA and incubated at 37 °C in a humidified CO_2 incubator for 1 h. The intracellular ROS production was measured by using an EnSpire® Multimode Plate Reader (PerkinElmer, MA, USA) with excitation wavelength at 485 nm and emission wavelength at 530 nm.

2.7 Experimental animal ethical approval

All animal procedures study were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 and the World Health Organization Guidelines for Breeding and Care of Laboratory Animals and approved by the committee of Centre for Animal Research, Naresuan University (NU-AE620615) and Chulalongkorn University Laboratory Animal Centre (CULAC), Protocol No.1973025. All surgery was performed under isoflurane combined with oxygen and all effort were made to minimize pain, suffering, and distress of the animals involved.

A total of 70 adults male Wistar rats (8 weeks old, 200-250 g) were purchased from Nomura Siam International, Bangkok, Thailand, which used for 14 rats in each group. These was divided into 2 subgroups including 8 for infarct size determination and cardiac function, and 6 for biochemical analysis and 14 for spare). Animals were maintained in an environmentally control condition (22°C ± 1°C, 12:12 h light: dark cycle) at the Chulalongkorn University Laboratory Animal Centre (CULAC), Bangkok, Thailand.

2.8 Experimental group assignment

Schematic diagram of experimental protocol, as shown in Figure 17. seventy adults male Wistar rats were used in this study, which divided into 2 experimental groups.

For physiological study, forty rats were used (Sham =8, Vehicle = 8, Treatment group =8 x2, 8 rats as spare animals). For biochemical analysis, thirty rat were used (Sham =6, Vehicle = 6, Treatment group =6 x2, 6 rat as spare animals). Wild-type SLPI (wt-SLPI, Sino Biology Inc., Beijing, China) was diluted in sterile phosphate buffered saline (PBS) and rat was injected through intravenous route with 50 µg of SLPI in 500 µl total solution at onset of the reperfusion. The dosage of SLPI in this study based on the previous reports in an *in vivo* model (313, 314). Similarly, the antiprotease-deficiency SLPI or mutated-SLPI (mt-SLPI) was administrated through intravenous route with a same dosage to wt-SLPI.

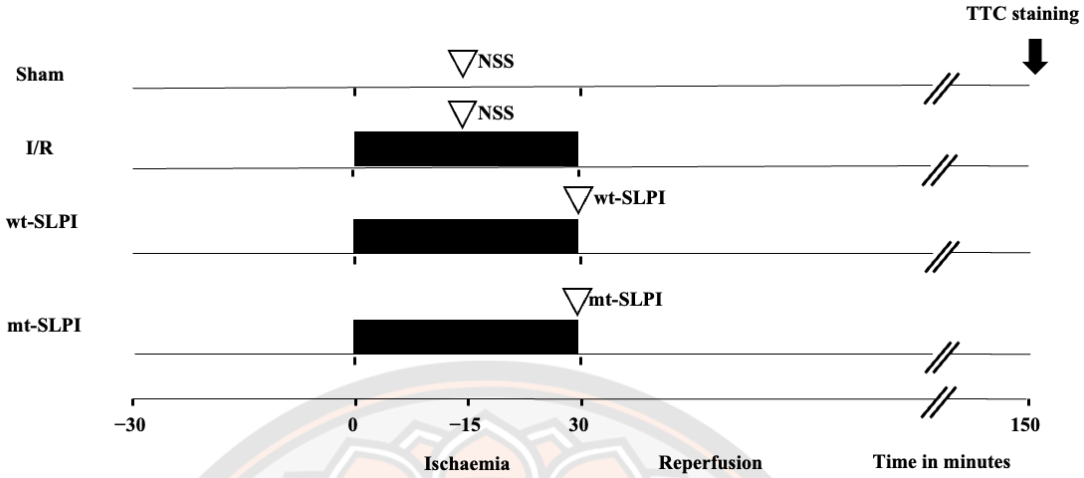


Figure 17 Schematic diagram of experimental protocol

2.9 Surgical preparation of myocardial I/R model in rats

The surgical procedure in this study was performed as described previously (308). Isoflurane induction chamber was used for anesthetized and followed by tracheotomy for intubation and connected to the rodent ventilator (VentElite, Harvard apparatus, MA, USA). The tidal volume was set at 2.5 mL with respiratory rate of 80 breaths/min and maintained with 1.8-2.0% isoflurane in 100% oxygen. The body temperature was maintained with warm water heating pump at 37°C throughout the surgery. Moreover, lead II electrocardiogram (ECG) was monitored by attached to the rat's chest The carotid artery was cannulated with Mikro-Tip® pressure catheter for rat (Millar, Houston, Texas, USA) for measuring left ventricular pressure (LVP) parameters. Blood samples were collected from fluid-filled catheter that canulated at jugular vein. In addition, jugular vein canulation was also used for administering the wt-SLPI and mt-SLPI. Then, left coronary artery was approached through left thoracotomy. Left anterior descending coronary artery (LAD) was tied with 6-0 synthetic, monofilament, nonabsorbable polypropylene suture over the PE-50 tube to simulate ischaemia. After ligation, an ischaemic condition was confirmed with an elevation of ST-segment in Lead II tracing of ECG and visual inspection as faded whitish colour of the epicardium below the ligation. The ligation was released after 30 min of occlusion by pulling the PE-50 out from the ligation while the suture was remained at the same place. The reperfusion was continued until 120 min after the beginning of reperfusion. The LVP was analysed for end-diastolic pressure (EDP), end-systolic pressure (ESP), develop pressure (devP), and heart rate (HR). The contractility was inferred from the maximum rate of rise of the LVP (dP/dt_{max}) and the contractility index (CI) defined as the dP/dt_{max} over the pressure at that point. In addition, the relaxation indices were determined by the maximum rate of fall of the LVP (dP/dt_{min}) and Tau. At the end of reperfusion period, the heart was quickly harvested and performed infarct size and area at risk experiments.

2.10 Evaluation of infarct size and area at risk

The evaluation of infarction and area at risk was performed at the end of surgery reperfusion period by re-occlusion the LAD as described previously (308). The area at risk (non-perfusion area during LAD ligation) was differentiated from non-infarction area by using 2%(w/v) Evans blue solution in PBS that injected via intravenous. Then, heart weight was measured and performed transversely heart slice section to 1 mm-thickness. The heart sections were incubated in 1% (w/v) of 2,3,5-triphenyl tetrazolium chloride (TTC) at 37 ˚C for 10 min for indicating necrotic myocardium. The sections were enhanced the contrast of tissues section by incubated in 10% (v/v) formalin for 15-20 h. The non-ischaemic area (Evans blue stained area) and infarct area (TTC negative) were blindly groups data and evaluated by using ImageJ software.

2.11 Determination of serum creatine kinase (MB isoenzyme) and lactate dehydrogenase (LDH) activity

At the end of the animal protocol, blood collection was performed and spined down for collecting the blood serum. The levels of biomarkers including creatine kinase (MB isoenzyme) and lactate dehydrogenase (LDH) activity was evaluated and analysed by automate biochemistry analyser (Cobas c 111 analysers, Roche)

2.12 Tissue Homogenate and protein collection

Another set of experimental animals (n = 6 for each group) was subjected to I/R injury, similar to the procedure of the experiment for infarct size determination and cardiac function, and animals were treated wt-SLPI or mt-SLPI. After the surgery, the heart was excised immediately at the end of experiment. The ventricle part was collected and quickly frozen in liquid nitrogen and -80 ˚C until performed next experiment. One hundred milligrams of ventricle tissue were homogenized in 1 mL of homogenization buffer (20 mM Tris HCl pH 6.8, 1 mM Na_3VO_4, 5 mM NaF and cOmplete™ Protease Inhibitor Cocktail (Merck Millipore, USA)). The tissues were gently homogenized by using hand homogenizer on ice.

Tissue homogenate was then collected and centrifuged at 14000 rpm for 10 min at 4 ˚C. The supernatant was collected and frozen for further experiment.

2.13 Determination of protein concentration by Bradford assay

Protein concentration was measured by using Bradford reagent (HIMEDIA®, India). Five microliter of protein extract was mixed with 250 µL of Bradford reagent, which then incubated at room temperature for 5 min and measured at the absorbance at λ595 nm by spectrophotometer. Protein concentration was calculated and normalized by standard curve that created from bovine serum albumin (BSA). The protein concentration was used for fixing the amount of total protein to 100 µg and 25 µg that using in ELISA and Western blot analysis, respectively.

2.14 Western blot analysis

The heart homogenates were mixed with an equal volume of 2×SDS-PAGE sample buffers, containing 10% (v/v) 2-mercaptoethanol and bromophenol blue dye. Then, the samples were boiled for 10 min. Heart homogenate protein was separated on 10 or 12% SDS-polyacrylamide gels and transferred into Immobilon-P membranes (Millipore). Membranes were incubated in 5% (w/v) dried skimmed milk powder in Tris-buffered saline (pH 7.4) containing 0.1% Triton X-100 (TBST) buffer for 1 h at room temperature. After that, membrane were probed overnight at 4 °C with the appropriate primary antibody as follows: phosphorylated p38 (Cell signalling Technology, Danvers, MA, USA, #9211), total-p38 (Cell signalling Technology, Danvers, MA, USA, #8690), Bax (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, #sc-493), cleaved-caspase 3 (Cell signalling Technology, Danvers, MA, USA, #9661) and caspase-8 (Cell signalling Technology, Danvers, MA, USA, #4790), Actin (Cell signalling Technology, Danvers, MA, USA, #4967) (all of the primary antibodies were diluted at 1:1000 in 1% (w/v) skimmed milk + TBST buffer). Then, the membranes were washed and exposed for 1 h at room temperature to horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit (Merck, Darmstadt, Germany, #AP132P) at 1:5000 in 1% (w/v) skimmed milk in TBST buffer. The signal was developed by exposure the membranes with Affinity® ECL Western Blot Kit, picogram Grade (Affinity Biosciences, Melbourne,

Australia). The band intensity quantitation was captured and analysed by using ImageQuant™ LAS 500 (GE Healthcare, Chicago, IL, USA).

Data analysis

All experiments were performed in triplicate. Data was processed using SPSS (Statistical Package for Social Sciences) release 13.0 for Windows and reported as mean ± standard error (SEM). All comparisons involving more than one group was assessed for significance using one-way analysis of variance (ANOVA), followed when appropriate by the Tukey-Kramer test. Some parameters, which were compared between more than one groups or at the different time points, were assessed for significance using two-way analysis of variance (ANOVA). The p value < 0.05 was considered as significant. The statistical tests were be performed using commercially available software (Graph pad prism version 5).

CHAPTER IV

RESULTS

The results in this study were divided into 2 parts including

1) The determination of post-ischaemic treatment of recombinant human SLPI treatment on rat hearts subjected to an *in vivo* ischaemia/reperfusion.

2) The effect of antiprotease-deficiency of SLPI on ischaemia/reperfusion injury.

Part 1: Determination of post-ischaemic treatment of recombinant human SLPI treatment on rat hearts subjected to an *in vivo* ischaemia/reperfusion.

1.1 The Animal Body Weight and Heart Weight of Experimental Animals

The animal body weight was measured before the surgery and heart weight was measured after the end of surgical procedures. The results showed that the body weight of all groups was not significantly different (355.1 ± 8.21 g, 346.2 ± 5.77 g, 362.6 ± 8.544 g and 374.1 ± 10.31 g, $p > 0.05$) (Figure 18A) Similarly, the heart weight of rats in all groups was also not significantly different (0.893 ± 0.03 g, 0.886 ± 0.05 g, 0.960 ± 0.04 g and 0.9335 ± 0.03 g, $p > 0.05$) (Figure 18B). Therefore, the calculated heart weight and body weight ratio (HW/BW) also showed no significant difference in every group (0.0026 ± 0.0006, 0.0027 ± 0.0009 *vs.* 0.0026 ± 0.0001 and 0.0025 ± 0.0005, $p > 0.05$) (Figure 18C).

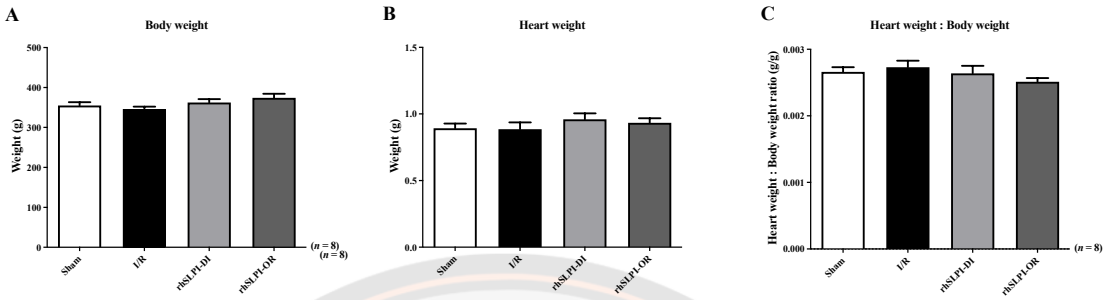


Figure 18 Determination of body weight of experimental animal before performing the surgical procedure of myocardial I/R injury (A), heart weight harvested at the end of the surgery (B) and heart weight to body weight ratio in rats of the study cohort (C).

1.2 Effect of Post-Ischemic rhSLPI Treatment on Infarct Size

First, we confirmed by evaluating the area at risk (AAR) that there was no difference in the I/R injury induced in all rats. Indeed, AAR was 49.38 ± 1.72, 47.64 ± 3.85 and 46.89 ± 5.93 in I/R, rhSLPI-DI and rhSLPI-OR, respectively (Figure 19A). The results in Figure 20B showed that infarct size (% infarct/AAR) in rats treated by rhSLPI was significantly lower than that of the non-treated I/R group (34.53 ± 2.61% for rhSLPI-DI and 31.75 ± 4.64% for rhSLPI-OR *vs.* 47.32 ± 3.83% for I/R, $p < 0.05$) (Figure 19B).

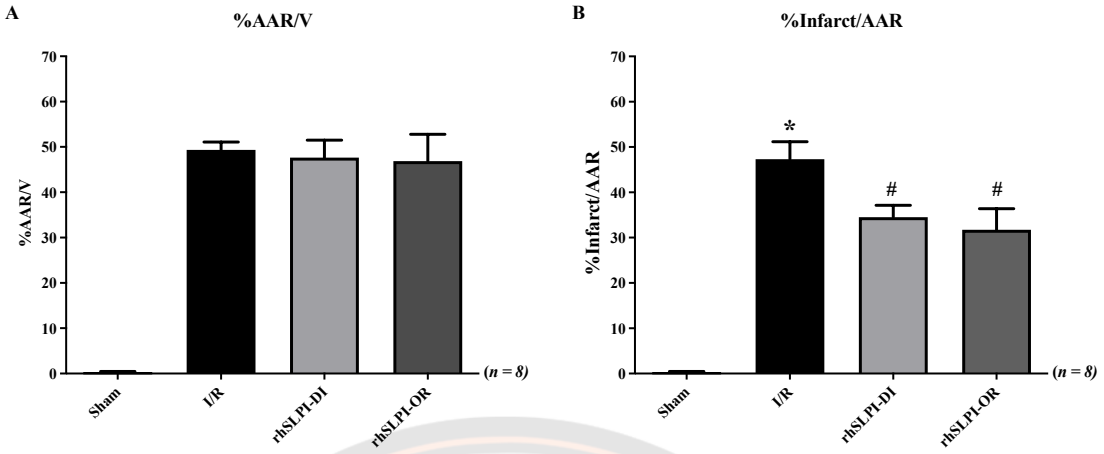


Figure 19 Determination of the percentage of area at risk (AAR) to ventricle volume (V) (A), percentage of infarct size to area at risk (B), (* $p < 0.05$ *vs.* sham) ($^{\#}$ $p < 0.05$ *vs.* I/R)

1.3 Effect of Post-Ischemic rhSLPI Treatment on Cardiac Biomarkers

Serum cardiac biomarkers were assessed by measuring both LDH and CK-MB activity.

The results showed that the LDH activity of the I/R group was significantly higher than that of the sham group (664.9 ± 118.9 *vs.* 218.4 ± 40.79 unit per litter (U/L), $p < 0.05$) (Figure 20A). Treatment with rhSLPI significantly reduced serum LDH activity in both the rhSLPI-DI group and the rhSLPI-OR group compared to that of the I/R group (363.0 ± 79.91 U/L, 299.8 ± 150.2 U/L *vs.* 664.9 ± 118.9 U/L, $p < 0.05$) (Figure 20A).

Similarly, for CM-MB activity, values obtained for the I/R group were significantly higher than those of the sham group (203 ± 13.43 U/L *vs.* 14.8 ± 11.20 U/L, $p < 0.05$) (Figure 20B). Treatment with rhSLPI significantly reduced serum CK-MB activity in both treated groups *vs.* the I/R group (141.0 ± 5.27 U/L and 138.8 ± 10.08 U/L *vs.* 203.8 ± 13.41 U/L, $p < 0.05$) (Figure 20B).

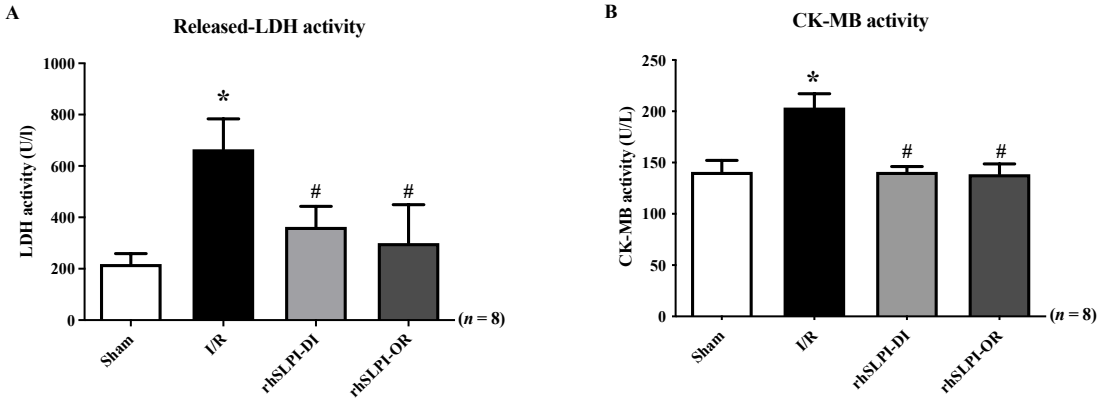


Figure 20 Determination of the activity of Lactate Dehydrogenase (LDH) (A) and Creatine kinase-MB (CK-MB) (B) (* $p < 0.05$ *vs.* sham) ($^{\#}$ $p < 0.05$ *vs.* I/R)

1.4 Effect of rhSLPI Treatment on LVP in I/R Rat

The LVP of the I/R rat was evaluated by using high-fidelity micromanometer catheters placed inside the left ventricular chamber. The results were divided into three phases including at baseline (after stabilization for 30 min prior to LAD ligation), ischemia (30 min after LAD ligation) and reperfusion (at 120 min after reperfusion). At the baseline phase, all of the LVP parameters including the EDP, ESP, dP/dt_{max}, CtrI, dP/dt_{min}, Tau/e, devP and HR were not significantly different when compared among groups (Table 2) The LVP parameters of the ischemic phase (30 min after LAD ligation) are shown in Table 3. The result shows that ischemia induced a reduction in ESP, dP/dt_{max} and devP and an increase in EDP, dp/dt_{min} and Tau/e. Treatment with rhSLPI during ischemia significantly reduced EDP, dp/dt_{min} and Tau/e and increased ESP, dP/dt_{max} and devP. The LVP parameters in the reperfusion phase (at 120 min after reperfusion) are shown in Table 4. The result shows ischemia induced a reduction in ESP, dP/dt_{max} and devP and an increase in EDP, dp/dt_{min} and Tau/e. Treatment with rhSLPI both during ischemia and at the onset of reperfusion significantly reduced EDP, dp/dt_{min} and Tau/e and increased ESP, dP/dt_{max} and devP.

Table 2 The baseline parameters of left ventricular pressure (LVP) determined the effect of rhSLPI on the LVP parameters in I/R (baseline).

Parameters	Group			
	Sham (*n* = 8)	**I/R (*n* = 8)**	**rhSLPI-DI (*n* = 8)**	**rhSLPI-OR (*n* = 8)**
EDP (mmHg)	4.87 ± 0.53	4.23 ± 0.16	4.43 ± 0.53	4.75 ± 0.29
ESP (mmHg)	113.8 ± 6.34	104.80 ± 9.31	119.2 ± 15.30	118.9 ± 17.25
dp/dt_{max} (mmHg/s)	5233 ± 359.2	5071 ± 391.1	6113 ± 575.4	5470 ± 287.1
CtrI	100.5 ± 2.02	106.9 ± 5.59	99.73 ± 7.59	100.4 ± 6.0
dp/dt_{min} (mmHg/s)	−4989 ± 383.2	−4526 ± 487.1	−5627 ± 831.8	−5116 ± 362.6
Tau/e (ms)	10.18 ± 0.21	11.03 ± 0.43	9.67 ± 0.83	9.34 ± 0.73
devP (mmHg)	109.4 ± 5.40	108.1 ± 9.85	115.8 ± 16.75	109.8 ± 12.96
HR (bpm)	431.5 ± 18.09	426.9 ± 20.09	416.4 ± 10.04	437.8 ± 13.66

*$p < 0.05$ *vs.* sham, $^{\#}$ $p < 0.05$ *vs.* I/R

Table 3 The parameters of left ventricular pressure of ischemic phase determined the effect of rhSLPI on LVP parameters in MI (30 min after ligation).

Parameters	Group			
	Sham (*n* = 8)	**I/R (*n* = 8)**	**rhSLPI DI (*n* = 8)**	**rhSLPI-OR (*n* = 8)**
EDP (mmHg)	5.36 ± 0.40	8.63 ± 1.55 *	3.65 ± 0.46 #	7.29 ± 0.18
ESP (mmHg)	111.8 ± 10.1	81.81 ± 9.69 *	131.2 ± 9.08 #	101.8 ± 17.0
dp/dt_{max} (mmHg/s)	5072 ± 725.1	3547 ± 415.7 *	5983 ± 373.3 #	4520 ± 541.0
CtrI	97.63 ± 5.21	101.5 ± 6.60	100.6 ± 2.55	80.88 ± 21.69
dp/dt_{min} (mmHg/s)	−4185 ± 206.6	−3139 ± 93.69 *	−5646 ± 701.1 #	−4565 ± 590.5
Tau/e (ms)	10.11 ± 0.46	12.38 ± 1.05 *	10.04 ± 0.37 #	8.744 ± 0.22
devP (mmHg)	106.9 ± 9.78	83.47 ± 7.72 *	126.7 ± 12.82 #	105.5 ± 8.85
HR (bpm)	429.3 ± 19.13	405.3 ± 24.52	390.1 ± 15.31	421.2 ± 12.75

* $p < 0.05$ *vs.* sham, # $p < 0.05$ *vs.* I/R.

Table 4 The parameters of left ventricular pressure of reperfusion phase determined the rhSLPI effect on LVP parameters in MI (120 min after reperfusion).

Parameters	Group			
	Sham (*n* = 8)	**I/R (*n* = 8)**	**rhSLPI-DI (*n* = 8)**	**rhSLPI-OR (*n* = 8)**
EDP (mmHg)	5.85 ± 0.60	7.63 ± 0.08 *	2.871 ± 0.19 #	4.084 ± 0.60 #
ESP (mmHg)	105.6 ± 6.65	82.74 ± 6.63 *	111.1 ± 10.01 #	94.47 ± 8.80 #
dp/dt_{max} (mmHg/s)	4509 ± 527.4	2836 ± 373.4 *	4739 ± 466.8 #	4458 ± 376 #
CtrI	91.27 ± 5.79	94.41 ± 9.65	90.42 ± 2.79	72.85 ± 20.30
dp/dt_{min} (mmHg/s)	−4890 ± 569.3	−2418 ± 669.7 *	−4127 ± 244.6 #	−3990 ± 264.3 #
Tau/e (ms)	11.24 ± 0.53	14.83 ± 3.58	9.73 ± 0.54	8.754 ± 1.37
devP (mmHg)	104.4 ± 7.40	80.96 ± 7.25	96.28 ± 4.08	95.51 ± 6.61
HR (Bpm)	408.5 ± 20.81	373.9 ± 42.74	402.7 ± 6.47	410.3 ± 10.92

* $p < 0.05$ *vs.* sham, # $p < 0.05$ *vs.* I/R.

1.5 Effect of Post-Ischemic rhSLPI Treatment on Inflammatory Cytokines Level

Excessive inflammation has been described as one of the pathophysiological mechanisms that occurs in myocardial I/R injury and plays a key role in inducing cell death. In this study, the cardiac inflammatory cytokines level including IL-1β, IL-6 and TNF-α was measured by ELISA. The results show that there was a marked increase in the cardiac IL-1β level in the I/R group vs. the sham group (309.3 ± 32.45 ng/mL *vs.* 221.4 ± 16.91 ng/mL, $p < 0.05$) (Figure 21A). The rhSLPI treatment at reperfusion was able to drastically decrease the IL-1β level while rhSLPI treatment during ischemia was not. There was no difference in the I/R group vs. sham for the cardiac levels of IL-6 and TNF-α (IL-6: 332.1 ± 16.72 ng/mL *vs.* 328.9 ± 11.45 ng/mL, and TNF-α: 256.7 ± 18.68 ng/mL *vs.* 229.9 ± 5.02 ng/mL, $p < 0.05$) (Figure 21B,C). However, post-ischemic treatments with rhSLPI significantly reduced the cardiac IL-6 level when compared to the I/R group (267.3 ± 18.34 ng/mL *vs.* 332.1 ± 16.73 ng/mL, $p < 0.05$) (Figure 21B). Interestingly, the results show that cardiac IL-1β, IL-6 and TNF-α levels in the rhSLPI-OR group were significantly lower than the I/R group (Figure 21A-C).

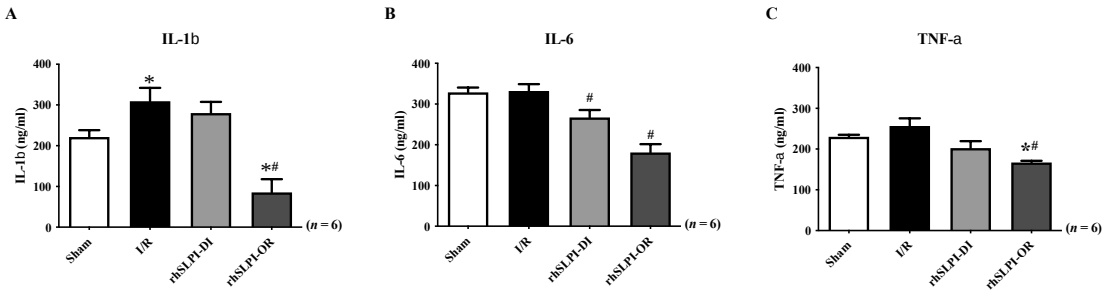


Figure 21 Determination of effect of post-ischaemic treatment of rhSLPI on cardiac inflammatory cytokines level including IL-1β (A), IL-6 (B) and TNF-α (C) in the sham group, I/R group, rhSLPI-DI and rhSLPI-OR (* $p < 0.05$ *vs.* sham) ($^{\#}$ $p < 0.05$ *vs.* I/R) (n = 6).

1.6 Effect of Post-Ischemic rhSLPI Treatment on Oxidatively cardiac and serum protein carbonylation level

The result shows that cardiac PC content levels in the I/R group were significantly higher than those of the sham group (cardiac PC: 70.75 ± 2.40 nmol/mg vs. 43.70 ± 0.78 nmol/mg, and serum PC: 3.66 ± 0.15 *vs.* 2.95 ± 0.31, $p < 0.05$) (Figure 22A,B). Treatment with rhSLPI in rhSLPI at reperfusion was significantly lower than that of the I/R group (49.08 ± 75.70 nmol/mg *vs.* 70.75 ± 2.40 nmol/mg, $p < 0.05$) (Figure 22A). In addition, the result shows that the serum PC content level in rhSLPI-DI and rhSLPI-OR was significantly lower than that of the I/R group (3.25 ± 0.04 nmol/mg and 3.04 ± 0.11 nmol/mg *vs.* 3.66 ± 0.15 nmol/mg, $p < 0.05$)

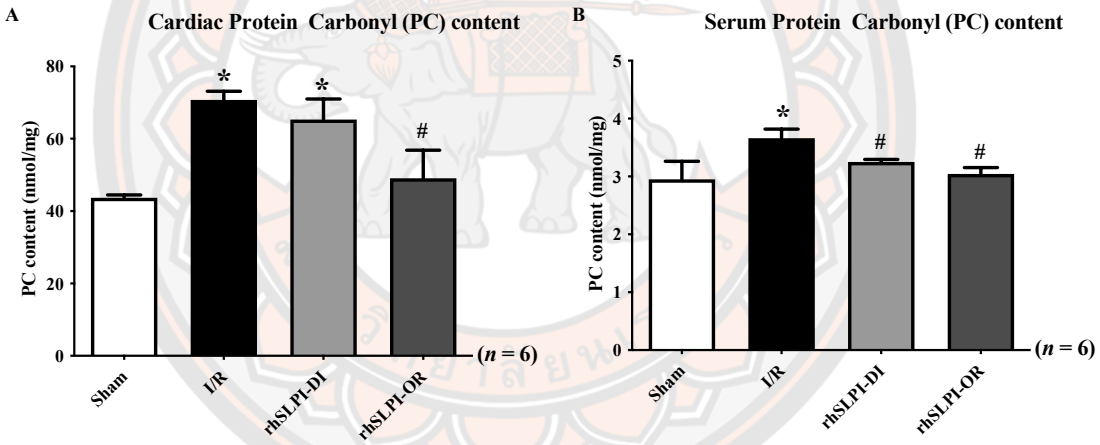


Figure 22 Determination of cardiac(A) and serum(B) protein carbonylation after treated with rhSLPI-DI and rhSLPI-OR (* $p < 0.05$ *vs.* sham) ($^{\#}$ $p < 0.05$ *vs.* I/R) (n = 6).

1.7 Effect of Post-Ischemic rhSLPI Treatment on Oxidatively cardiac and serum ischaemic modified albumin (IMA) level

In addition, the results show that the cardiac IMA level in I/R, rhSLPI DI and rhSLPI-OR groups was significantly higher than that of the sham group (0.24 ± 0.04, 0.24 ± 0.03 and 0.24 ± 0.03 arbitrary unit (A.U.) *vs.* 0.16 ± 0.01 A.U., $p < 0.05$) (Figure 23A). However, the serum IMA level of the I/R group showed a trend to be greater than the sham group, but this was not statistically significant (0.17 ± 0.01 A.U. *vs.* 0.15 ± 01 A.U., $p > 0.05$) (Figure 23B); however, treatment with rhSLPI in the rhSLPI-DI group showed, in serum, a significantly lower IMA level than that of the I/R group (0.17 ± 0.01 A.U. *vs.* 0.15 ± 0.01 A.U., $p < 0.05$) (Figure 23B).

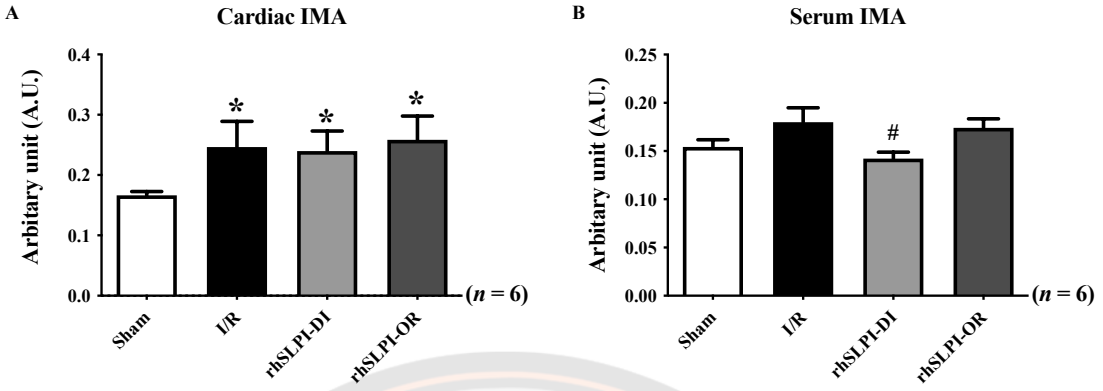


Figure 23 Determination of cardiac (A) and serum (B) ischaemic modified albumin level after treated with rhSLPI-DI and rhSLPI-OR (* $p < 0.05$ *vs.* sham) ($^{\#}$ $p < 0.05$ *vs.* I/R) (n = 6)

1.8 Effect of Post-Ischemic rhSLPI Treatment on Signal Transduction Molecules

To determine the effect of rhSLPI in signal transduction on the organ level response to myocardial ischemia/reperfusion injury, Western blot analysis was performed on the heart homogenate protein. The results show that there was a significant activation of p38 MAPK and JNK phosphorylation in the I/R group, when compared to the sham group (p38 MAPK: 1.07 ± 0.18 *vs.* 0.50 ± 0.07, and JNK: 0.23 ± 0.012 *vs.* 0.14 ± 0.01, $p < 0.05$) (Figure 24A-C). Similarly, ERK and Akt phosphorylation was significantly lower in the I/R group, when compared to the sham group (ERK: 0.26 ± 0.04 *vs.*0.39 ± 0.03, and Akt: 0.41 ± 0.07 *vs.* 0.74 ± 0.15, $p < 0.05$) (Figure 24A,D,E). In post-ischemic rhSLPI treatment groups, showed that rhSLPI could significantly reduce p38 MAPK activation (0.57 ± 0.06 and 0.49 ± 0.09 *vs.* 1.07 ± 0.18, $p < 0.05$) (Figure 24B). In contrast, rhSLPI could enhance JNK, ERK and Akt phosphorylation when compared with the I/R group (0.28 ± 0.01 *vs.* 0.23 ±0.01, 0.54 ± 0.09 *vs.* 0.26 ± 0.04 and 0.77 ± 0.06 *vs.* 0.41 ± 0.07, $p < 0.05$).

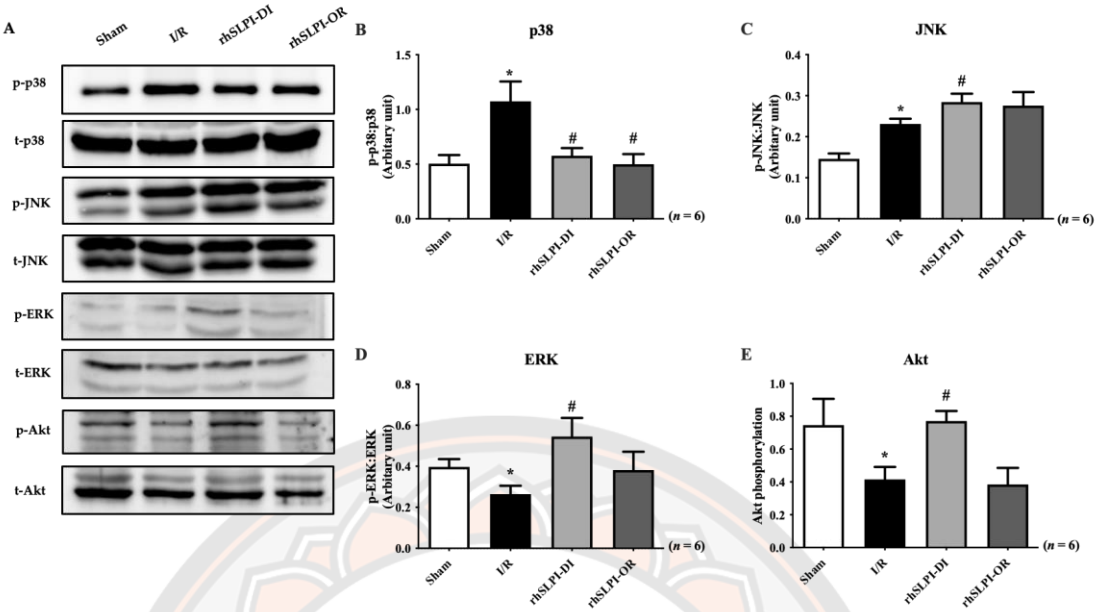


Figure 24 Determination of rhSLPI on activation of MAPK kinase involved in cell injury and survival in MI rat by Western blot analysis (A). The quantitative analysis for the MAPK phosphorylation was presented for p38 (B), JNK (C), ERK (D) and Akt (E). *(* $p < 0.05$ vs.* sham). (# $p < 0.05$ *vs.* I/R) (n = 6)

1.9 Effect of Post-Ischemic rhSLPI Treatment on Apoptosis Regulatory Molecules

To determine the effect of rhSLPI on apoptosis regulatory molecules on the organ level response to myocardial ischemia/reperfusion injury, the results show that there was a significant increase in Bax, caspase-3 and caspase-8 levels of the I/R group, when compared to the sham group (Bax 0.37 ± 0.03 *vs.*0.11 ± 0.02, caspase-3 1.27 ± 0.27 *vs.* 0.45 ± 0.08 and caspase-8 0.83 ± 0.05 *vs.* 0.36 ± 0.04, $p < 0.05$) (Figure 25A-D). Moreover, treatment of rhSLPI at the onset at reperfusion showed significantly reduced activation of Bax, caspase-3 and capase-8 (0.16 ± 0.01 *vs.* 0.37 ± 0.03, 0.35 ± 0.04 *vs.* 1.27 ± 0.27 and 0.55 ± 0.10 *vs.* 0.83 ± 0.05, $p < 0.05$) (Figure 25B-D).

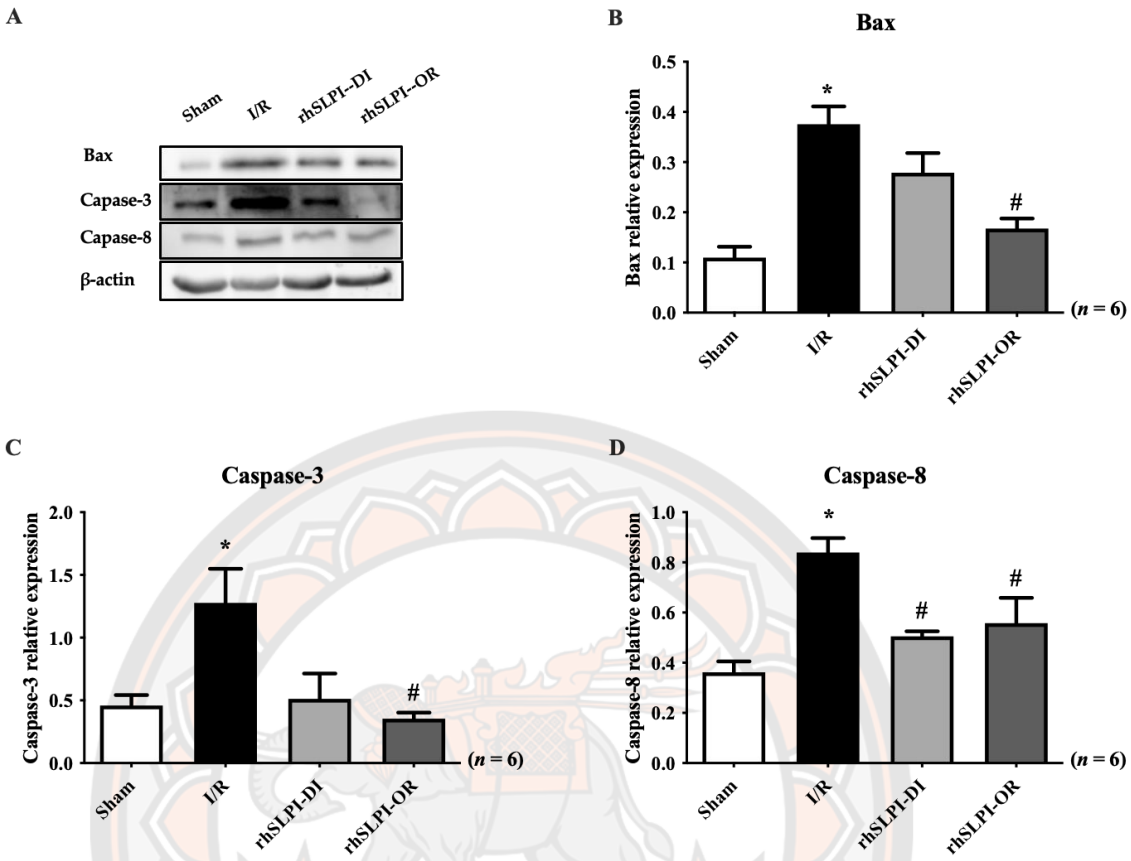


Figure 25 Determination of rhSLPI on apoptosis regulatory protein levels in MI rat by Western blot analysis (A). The quantitative analysis for the expression was presented for bax (B), caspase-3 (C) and caspase-8 (D) (* $p < 0.05$ *vs.* sham) (# $p < 0.05$ *vs.* I/R) (n = 6).

Part 2: The effect of antiprotease-deficiency of SLPI on I/R injury.

2.1 The neutrophil elastase activity of wild-type and mutated SLPI on H9c2 cells

The neutrophil elastase activity of wild-type(wt) and mutated(mt) SLPI were measured by fluorometric technique. The results showed that the enzymatic activity of NE was significantly inhibited when preincubated with wt-SLPI to 22.0 ± 2.51%RFU when compared to that of control ($p < 0.05$), whereas preincubated NE with mt-SLPI failed to inhibit the NE activity. (Figure 26).

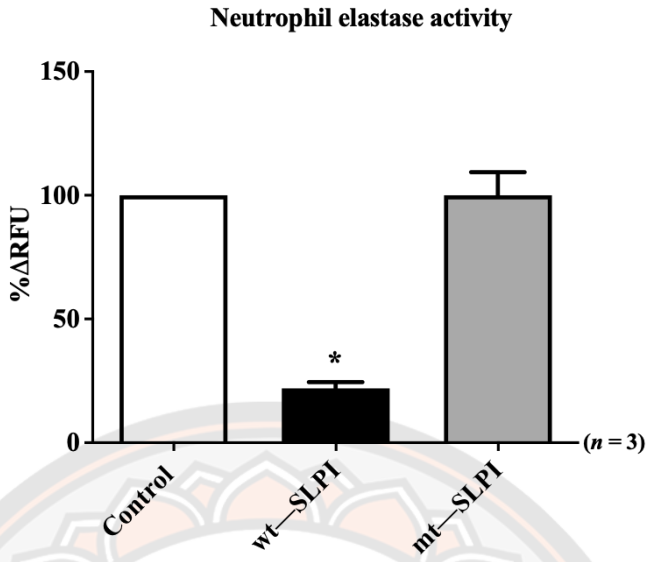


Figure 26 Determination of neutrophil elastase activity of wt-SLPI and mt-SLPI, *(*p* < 0.05 *vs.* control)

Then, both of wt-SLPI and mt-SLPI were treated to H9c2 and was determined for cellular toxicity. The results showed that there was not significantly difference in cell viability when cells exposed to both of wt-SLPI and mt-SLPI in comparison to those of control group (100 ± 2.44 and 100 ± 5.29 *vs.* 101.1 ± 2.03, $p > 0.05$) (Figure 27).

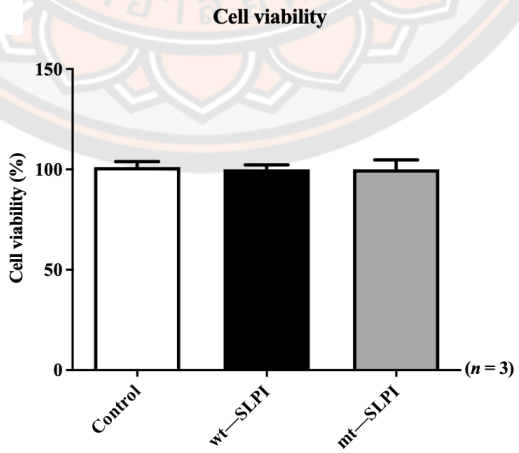


Figure 27 Determination of percentage of cell viability after treated with wt-SLPI and mt-SLPI (B) *(*p* < 0.05 *vs.* control)

2.2 Anti-protease deficient SLPI reduced cardiac cell injury and intracellular ROS production in hypoxia/reoxygenation(H/R) condition

An *in vitro* cardioprotective effect of mt-SLPI was performed against hypoxia/reoxygenation(H/R) condition. The result showed that mt-SLPI significantly increased cell viability when compared with H/R group (83.38 ± 3.57 *vs.* 70.05 ± 2.93, $p < 0.05$), which was comparable to wt-SLPI (83.16 ± 2.85 *vs.* 70.05 ± 2.93, $p < 0.05$) (Figure 28).

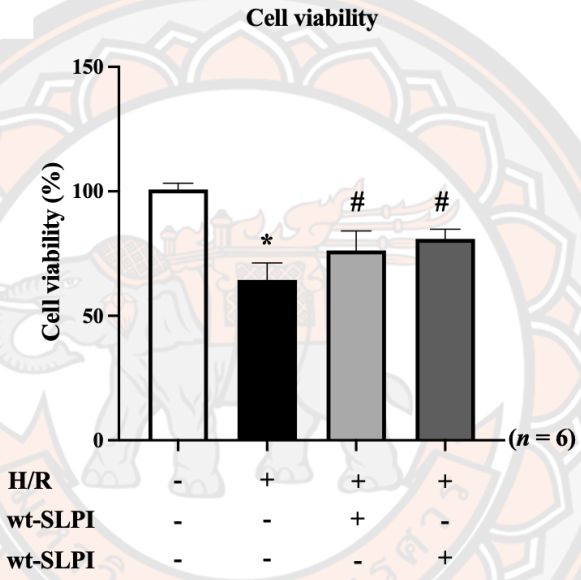


Figure 28 Determination of percentage of cell viability under H/R condition and treated with wt-SLPI and mt-SLPI (* $p < 0.05$ *vs.* control) ($^{\#}p < 0.05$ *vs.* H/R).

The intracellular ROS level was also determined. The results showed that treatment with both of wt-SLPI and mt-SLPI could significantly reduce intracellular ROS level when compared with H/R group (2,488,000 and 2,425,000 *vs.* 2,941,000 Arbitrary unit (A.U.), $p < 0.05$) (Figure 29)

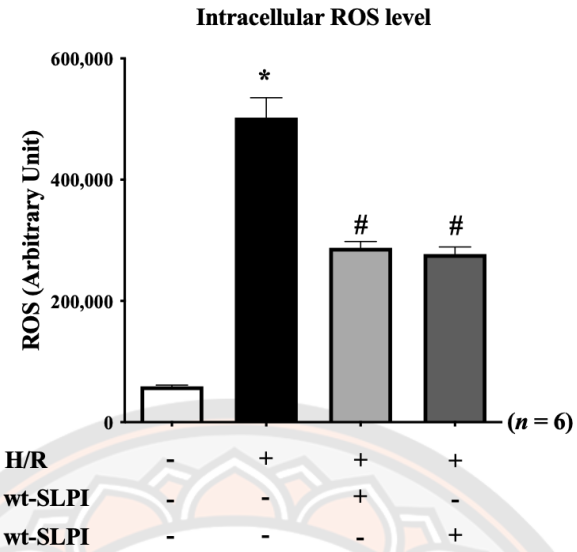


Figure 29 Determination of intracellular ROS under H/R condition and treated with wt-SLPI and mt-SLPI (* $p < 0.05$ *vs.* control) ($^{\#}p < 0.05$ *vs.* H/R).

2.3 Anti-protease deficient SLPI reduced infarct size cardiac biomarkers in an *in vivo* I/R injury.

There were not significantly difference in % area at risk (AAR) in I/R injury group and both treated groups (Figure 30A). Results in Figure 30B showed that infarct size (% infarct/AAR) in rats treated with both of wt-SLPI and mt-SLPI was significantly lower than that of I/R group (31.75 ± 4.64, 41.60 ± 1.98 *vs.* 52.65 ± 4.23, $p < 0.05$).

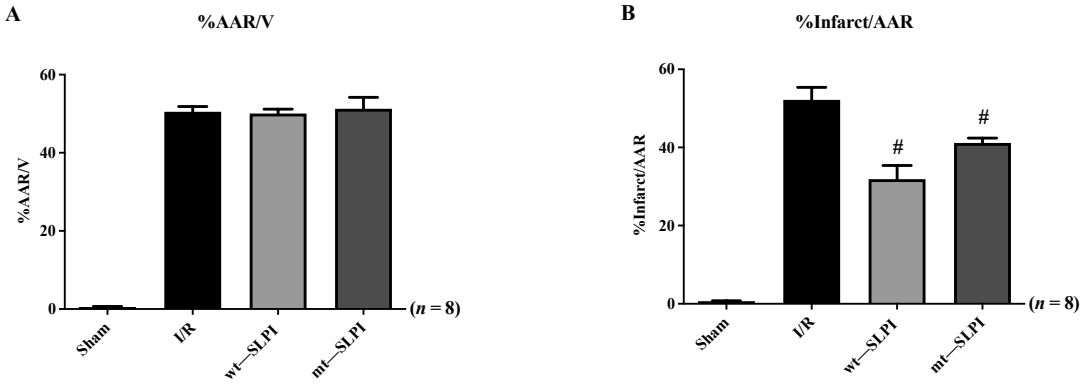


Figure 30 Determination of the percentage of area at risk(AAR) to ventricle volume(V) (A), percentage of infarct size to area at risk(B) (* $p < 0.05$ *vs.* sham) ($^{\#}$ $p < 0.05$ *vs.* I/R).

2.4 Anti-protease deficient SLPI reduced cardiac biomarkers in an *in vivo* I/R injury.

Cardiac biomarkers including LDH and CK-MB activity were measured from serum collected at the end of study protocol. The results showed that LDH activity of the I/R group was significantly higher than sham group (785.3 ± 162.1 *vs.* 187.8 ± 30.12 U/L, $p < 0.05$) (Figure 31A). Treatment group both of wt-SLPI and mt- SLPI was significantly reduced LDH activity than that of I/R group (364.5 ± 175.0 and 452.2 ± 43.43 *vs.* U/L, $p < 0.05$) (Figure 31A).

Similarly, there was a significant increase in CK-MB activity in I/R group when compared with sham group (213.1 ± 14.48 *vs.* 135.9 ± 11.42 U/L, $p < 0.05$) (Figure 31B). Treatment with both of wt-SLPI and mt-SLPI could significantly reduce CK-MB activity when compared with I/R group (138.8 ± 10.08 and 122.5 ± 7.34 *vs.* 213.1 ± 14.48 U/L, $p < 0.05$) (Figure 31B).

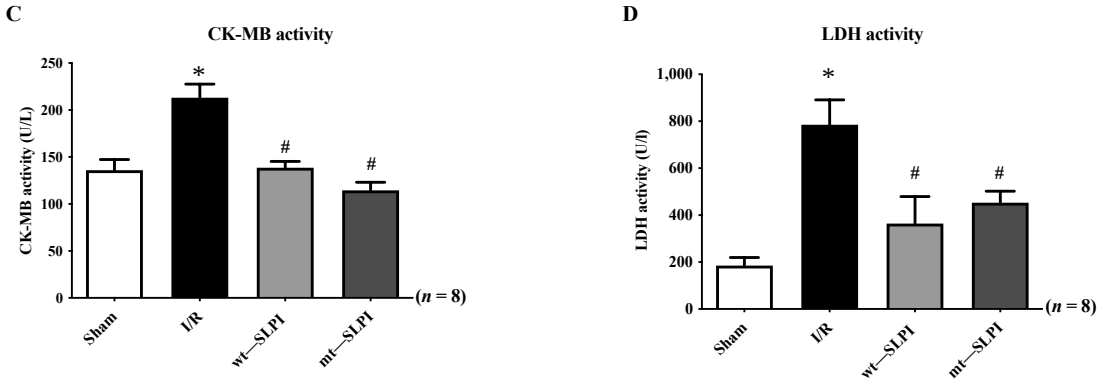


Figure 31 Determination of the activity of Lactate Dehydrogenase (LDH) (A) and Creatine kinase-MB (CK-MB) (B) (* $p < 0.05$ *vs.* sham) ($^{\#}$ $p < 0.05$ *vs.* I/R).

2.5 Effect of *in vivo* treatment of anti-protease deficient SLPI on cardiac function parameters in I/R injury

The left ventricular pressure (LVP) parameters were measured, and the data was collected by high-fidelity micromanometer catheters placed inside the left ventricular chamber. The results of LVP were divided into 3 phases according to the surgery processes including baseline (after stabilization for 30 min prior to LAD ligation), ischemia (30 min after LAD ligation) and reperfusion (at 120 min after reperfusion). At the baseline phase, all of LVP parameters including EDP, ESP, dP/dt_{max}, CI, dP/dt_{min}, Tau/e, devP and HR were not significantly different among groups (Table 5) At the ligation phase (30 min after LAD ligation), the ischaemia induced the reduction of ESP, dP/dt_{max}, devP and increased EDP, dP/dt_{min} and Tau/e in I/R, wt-SLPI, and mt-SLPI group when compared with sham group (Table 6). At the reperfusion phase, the I/R injury induced the reduction of ESP, $dP/dt_{max,}$ devP and increase of EDP, dP/dt_{min} and Tau/e when compared with sham group. After treatment with wt-SLPI, the results showed wt-SLPI could increase ESP, dP/dt_{max} , devP and reduced EDP, dP/dt_{min} and Tau/e when compared with I/R group (Table 7). Whereas, mt-SLPI treatment could reduce only EDP parameter when compared with I/R group.

Table 5 The baseline parameters of left ventricular pressure (LVP) determined the effect of SLPI on the LVP parameters in I/R (baseline).

Parameters	Group			
	Sham (*n*=8)	**I/R (*n*=8)**	**wt-SLPI (*n*=8)**	**mt-SLPI (*n*=8)**
EDP (mmHg)	5.07 ± 0.73	4.37 ± 0.12	4.70 ± 0.21	4.23 ± 0.20
ESP (mmHg)	118.7 ± 5.63	112.7 ± 7.15	122.2 ± 18.42	110.6 ± 6.17
dp/dt_{max} (mmHg/s)	4958 ± 328.00	4778 ± 367.30	5709 ± 226.00	4973 ± 662.60
CtrI	101.5 ± 2.47	104.4 ± 6.49	105.9 ± 3.29	103.6 ± 6.28
dp/dt_{min} (mmHg/s)	-4668 ± 295.00	-4884 ± 466.70	-4935 ±444.20	-4938 ± 336.00
Tau/e (ms)	10.32 ± 0.23	10.63 ± 0.24	8.657 ± 0.35	10.17 ± 0.11
devP (mmHg)	113.8 ± 4.41	116.2 ± 7.92	119.1 ± 11.59	110 ± 6.38
HR (bpm)	447.8 ± 11.13	438.3 ± 21.36	450.3 ± 7.02	450.2 ± 16.01

Table 6 The parameters of left ventricular pressure (LVP) of ischemic phase determined the effect of SLPI on the LVP parameters in I/R (ligation). * $p < 0.05$ *vs.* sham, $^{\#}$ $p < 0.05$ *vs.* I/R.

Parameters	**Group**			
	Sham (*n*=8)	**I/R (*n*=8)**	**wt-SLPI (*n*=8)**	**mt-SLPI (*n*=8)**
EDP (mmHg)	5.24 ± 0.28	10.10 ± 0.52 *	7.14 ± 0.10 *	6.72 ± 0.65 *
ESP (mmHg)	118.1 ± 11.26	90.83 ± 5.02 *	118.2 ± 4.65 *	102.5 ± 8.37 *
dp/dt_{max} (mmHg/s)	5517 ± 810.00	3244 ± 403.20 *	4902 ± 332.20 *	4454 ± 426.50 *
CtrI	99.86 ± 6.67	96.03 ± 4.80	107.8 ± 10.72	99.14 ± 4.15
dp/dt_{min} (mmHg/s)	-4267 ± 124.30	-3108 ± 61.71 *	-3689 ± 466.10 *	-3405 ± 496.40 *
Tau/e (ms)	9.713 ± 0.35	12.87 ± 1.20	8.686 ± 0.16	10.94 ± 0.46 *
devP (mmHg)	113 ± 6.84	79.55 ± 9.42 *	113.6 ± 4.69 *	96.51 ± 7.19 *
HR (bpm)	444.5 ±16.51	423.9 ± 20.68	432.4 ± 7.72	447.4 ± 9.24

Table 7 The baseline parameters of left ventricular pressure (LVP) of reperfusion phase determined the effect of SLPI on the LVP parameters in I/R (reperfusion). * $p < 0.05$ *vs.* sham, $^{\#}$ $p < 0.05$ *vs.* I/R.

Parameters	**Group**			
	Sham (*n*=8)	**I/R (*n*=8)**	**wt-SLPI (*n*=8)**	**mt-SLPI (*n*=8)**
EDP (mmHg)	6.20 ± 0.70	7.66 ± 0.06 *	4.22 ± 0.43 #	4.99 ± 0.52 #
ESP (mmHg)	109.7 ± 7.41	74.21 ± 7.09 *	104.5 ± 3.43 #	79.91 ± 8.71
dp/dt_{max} (mmHg/s)	4872 ± 541.80	2688 ± 419.70 *	4437 ± 484.60 #	3390 ± 338.10
CtrI	95.09 ± 6.07	80.05 ± 9.02	91.06 ± 11.58 #	93.65 ± 3.34
dp/dt_{min} (mmHg/s)	-5330 ± 510.10	-2252 ± 477.30 *	-3854 ± 585.90 #	-2922 ± 292.50
Tau/e (ms)	10.97 ± 0.64	15.94 ± 4.40 *	8.131 ± 1.58 #	12.57 ± 0.40
devP (mmHg)	106.6 ± 5.04	76.47 ± 8.06 *	101.1 ± 4.98 #	86.59 ± 6.40
HR (bpm)	425 ± 18.01	411.6 ± 26.03	417.9 ± 10.07	411.8 ± 10.92

2.6 Effect of anti-protease deficient SLPI on signal transduction and apoptosis regulatory molecules

The signal transduction that response to myocardial ischemia/reperfusion injury in the presence and absence of wt-SLPI and mt-SLPI at the onset of reperfusion was determined by Western blot analysis. The results showed that there was a significantly activation of p38 MAPK in I/R group when compared with sham group (1.07 ± 0.18 *vs.* 0.50 ± 0.07, $p < 0.05$) (Figure 32A). Treatment of both wt-SLPI and mt-SLPI at the onset of reperfusion could significantly reduce phosphorylated p38 MAPK when compared with I/R group (0.49 ± 0.09 and 0.50 ± 0.09 *vs.* 1.07 ± 0.18, $p < 0.05$). Similar results were observed in apoptotic regulatory pathway including Bax, caspase-3 and caspase-8. The results showed that I/R injury significantly increased pro-apoptotic proteins level when compared with sham group (Bax: 0.28 ± 0.04 *vs.* 0.11 ± 0.02, cleaved-Caspase-3: 0.49 ± 0.06 *vs.* 0.24 ± 0.02, Caspase-8: 0.79 ± 0.03 *vs.* 0.39 ± 0.03, $p < 0.05$) (Figure 32B, C and D). Treatment of both wt-SLPI and mt-SLPI significantly reduced the pro-apoptotic proteins level when compared with I/R group (Bax: 0.10 ± 0.02 and 0.15 ± 0.01 *vs.* 0.28 ± 0.04, cleaved-Caspase-3: 0.32 ± 0.04 and 0.35 ± 0.09 *vs.* 0.49 ± 0.06 Caspase-8: 0.46 ± 0.03 and 0.46 ± 0.03 *vs.* 0.79 ± 0.03, $p < 0.05$) (Figure 32B, C and D).

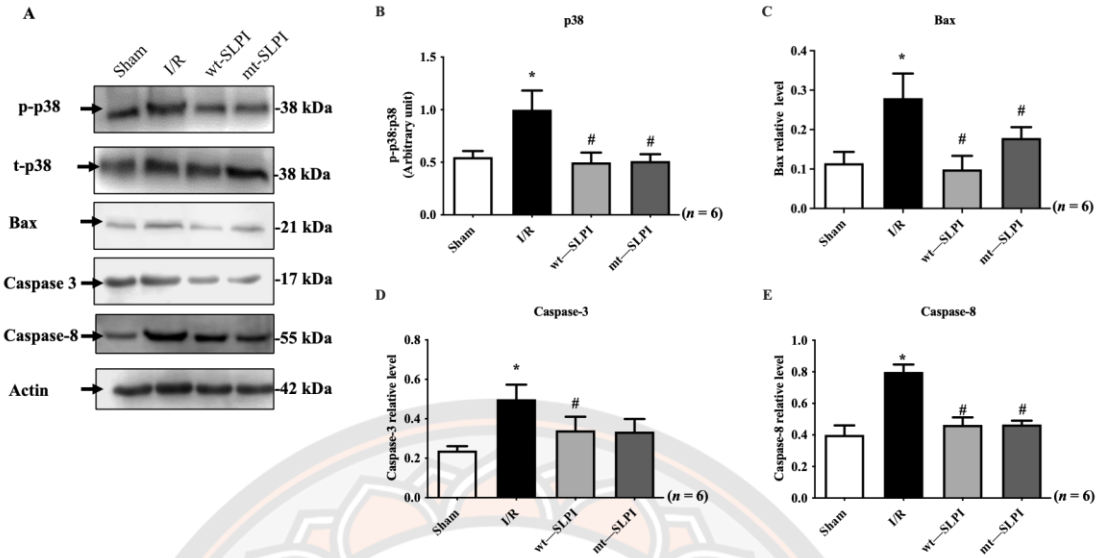


Figure 32 Determination of the apoptotic regulatory signalling protein level in MI rat by Western blot analysis (A). The quantitative analysis for the expression was presented for p38 MAPK phosphorylation (B), Bax (C), cleaved-Caspase-3 (D) and Caspase-8 (E) (* $p < 0.05$ *vs.* sham) ($^{\#}$ $p < 0.05$ *vs.* I/R) (n = 6)

CHAPTER V

DISCUSSION & CONCLUSION

In the present study, we demonstrated, for the first time, the cardioprotective effect of recombinant human secretory leukocyte protease inhibitor (rhSLPI) administered as a post-ischemic treatment in an *in vivo* rat model of myocardial I/R injury. The major findings of this study are that post-ischemic treatment with rhSLPI induced a reduction in infarct size, cardiac inflammatory cytokine levels and the production of oxidatively modified proteins and an improvement in cardiac function. Furthermore, rhSLPI treatment could be associated with the attenuation of apoptosis regulatory signalling, such as p38 MAPK, Bax, caspase-3 and caspase-8, and the activation of the cell survival proteins Akt and ERK by phosphorylation.

Proteases are crucial for the maintenance of cellular homeostasis through the destruction of adversely misfolded or malfunctioning proteins, extracellular matrix (ECM) degradation and activation of cellular processes (7, 11). In cardiomyocytes, protease enzymes are basically active but counterbalanced by their endogenous inhibitors in order to finely control their action (2). During myocardial I/R injury, the regulatory mechanisms are altered, and results in a large increase in both intracellular and extracellular protease activities (6-11). The leukocyte-derived inflammatory serine proteases (ISPs), both from neutrophil- and mast cell-derived serine proteases, such as calpain, matrix metalloproteinases (MMPs) and cathepsins, have been reported to be involved in cardiac injury (7). Alterations of protease activity not only cause cardiac cell injury and death but also result in subcellular cardiac remodeling and the loss of cardiac functions. Therefore, inhibition of protease activity can, therefore, be considered as a powerful strategy for preventing the expansion of injured tissue as well as the progression of cardiac remodeling and hypertrophy.

A strategy to inhibit protease activity, by using chymase inhibitor monotherapy, has been studied in several models, such as rodents, dogs and pigs, which provide similar information on reducing infarct size (94, 321-323). In addition, implementation of a dual-inhibitor (cathepsin and chymase inhibitor, DCCI) also

showed reduced infarction size (324). This evidence convinces the cardioprotective potential of serine protease inhibition against myocardial I/R injury. One of the concerning issues is that the protease inhibitors reported in those previous studies were small molecule chemical inhibitors. The findings from a computational study revealed the existence of chymase inhibitors of putative off-targets that were identified through various structural and functional similarity analyses as well as with molecular docking studies (97). This is a warning for possible adverse effects in the case of small molecule inhibitors. Alternatively, using broad endogenous anti-protease peptides, which are normally expressed and function in the body, could provide more reliable results and safety. Secretory leukocyte protease inhibitor (SLPI) is an endogenous anti-serine protease protein that is normally expressed in several mucous tissues. Several studies described its pharmacological properties including antimicrobial and antiviral (325), as well as anti-inflammatory (326) and anti-apoptotic activities (327). Our study demonstrates that the post-ischemic administration of rhSLPI as early as possible, even during ischemia, could reduce infarct size and improve cardiac function.

The ischemic condition impairs cardiac function by reducing hemodynamic parameters for both the systolic and diastolic functions. Treatment with rhSLPI either after the coronary ligation (during ischemia) or at the onset of reperfusion improved hemodynamic functions. This result correlates with reduced infarct size. An *in vivo* post-ischemic treatment with rhSLPI, injected at both time points, could significantly reduce infarct size. This finding is similar to that of our previous studies in both *in vitro* and *ex vivo* models of global ischemia (22, 23). The dose of rhSLPI in the present study (50 µg per 350 g body weight) is comparable to that used in our previous study (1000 ng/mL) which also provided cardioprotective effects (22).

Reactive oxygen species (ROS) are one of the detrimental components of I/R injury (328). ROS play a major role in inducing cell injury and cell death via directly damaging cell membranes and, indirectly, through the recruitment of inflammatory cells and activation of the pro-apoptotic pathways (147). It has been reported that SLPI could reduce intracellular ROS production in an in vitro simulated ischemia/reperfusion injury (22, 23, 307, 310, 311). In this study, the assessment of antioxidative stress was performed by determining the oxidatively modified products,

PC and IMA. It has been reported that PC was increased in patients with acute myocardial infarction (329) and heart failure (330). In addition, the IMA level was elevated in patients with acute coronary syndrome in all subgroups, including non-ischemic chest pain (NICP), unstable angina (UA) or myocardial infarction (MI) (331). Therefore, PC and IMA could be cardiac biomarkers for assessing the oxidative stress in myocardial I/R models. In this study, rhSLPI could reduce oxidative stress by lowering the protein carbonyl content level in both cardiac tissue and serum, which could explain the beneficial effect toward infarct size reduction. However, it seems that IMA levels were slightly modified by rhSLPI treatment. It is possibly due to the lower sensitivity of IMA in terms of modification its in terms of being a diagnostic marker. The previous study showed that, in comparison with PC, IMA showed lower diagnostic sensitivity and area under the receiver operating characteristic (ROC) for the diagnosis of non-ST elevation myocardial infarction patients (332).

One of the possible major effects leading to decreased cardiac injury, for the post-ischemic rhSLPI treatment, is the ability to reduce reperfusion-induced inflammation. Accordingly, the results of the present study show that post-ischemic rhSLPI treatment could reduce production of inflammatory cytokines IL-1β, TNF-α and IL-6. The anti-inflammatory effect of SLPI was demonstrated in the present study, corroborating the results obtained by Jin et al. showing that SLPI reduced inflammatory cytokines in activated macrophages (183, 333). The attenuation of inflammation, as well as the decrease in the infarct size, could also be associated with the reduction in p38 MAPK phosphorylation in the rhSLPI-treated groups. Although p38 MAPK and JNK are both major signalling pathways known to be involved in the inflammatory response and cardiac cell death in I/R injury (334), post-ischemic treatment with rhSLPI could only reduce p38 MAPK phosphorylation, not JNK. Our previous report also demonstrated *in vitro* that rhSLPI treatment showed an association with pro-survival kinase activation, i.e., protein kinase B (PKB) or Akt (22, 23). Akt is a serine/threonine protein kinase, which is involved in survival pathways similar to p44/p42 MAPK and ERK1/2. Similar to our previous findings, our study here demonstrates that an *in vivo* post-ischemic treatment of rhSLPI could restore Akt and ERK1/2 phosphorylation, which consequently attenuates apoptosis regulatory molecules, e.g., Bax, caspase-3 and caspase-8. Taken together, the findings

of our present study are consistent with our previous reports, using both an *in vitro* and an *ex vivo* study model. Indeed, rhSLPI treatment induced cardioprotection by reducing infarct size, by attenuating cardiac inflammation and oxidative stress and by its association with the regulatory molecules involved in cell death and injury, in order to finally result in an improved cardiac function (Figure 33).

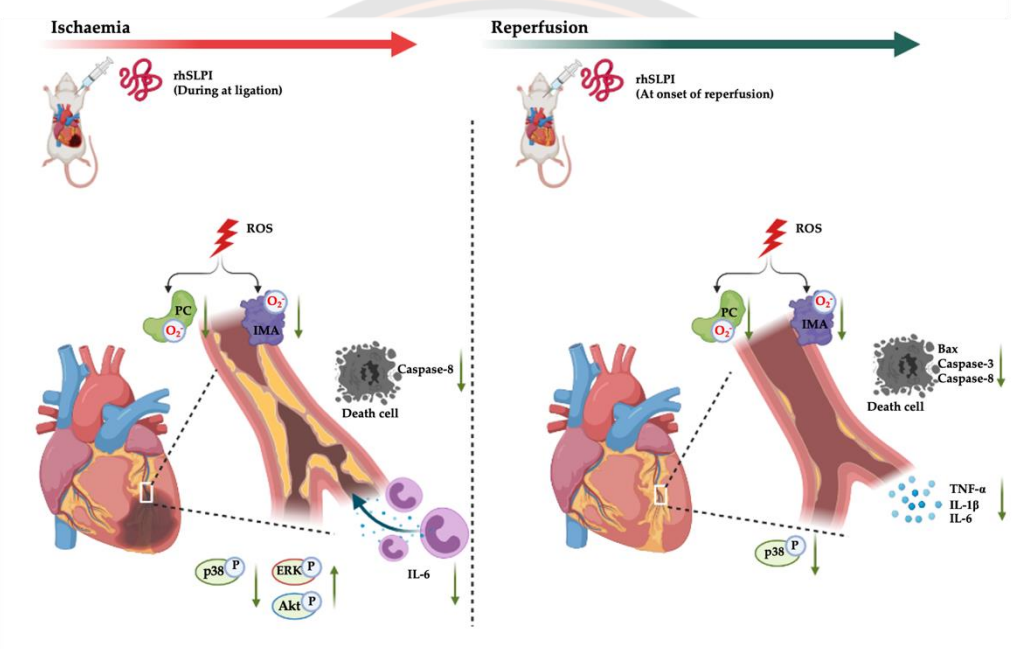


Figure 33 A schematic diagram of the major findings in this study. rhSLPI that was treated in the timing of the post-ischemia du-ration. rhSLPI could protect the heart from the ischemic condition. The mechanisms for this could be associated with the attenuation of intracellular ROS production, activation of Akt and p38 MAPK phosphorylation, reduction in inflammatory cytokines (TNF-α, IL-1β and IL-6) and reduction in apoptosis regulatory proteins (Bax, caspase-3, caspase-8).

The result of rhSLPI post-ischaemic treatment showed the potential of a cardioprotective effect in *in vivo* model that improved the cardiac function, and reduce infract size, cardiac markers, inflammation and apoptotic regulatory proteins. The cardioprotective effect of SLPI was believed to result of anti-protease activity. However, the mechanism of rhSLPI have not been clearly that due to anti-protease activity or direct effect of SLPI. Then, this is ***the first study*** to show the cardioprotective effect of anti-protease deficient SLPI in both an *in vitro*

hypoxia/reoxygenation in cardiac cells and administered as post-ischaemic treatment in a rat model subjected to an *in vivo* myocardial I/R injury. The major findings are that the mutant form of SLPI (mt-SLPI) containing triple substitutions of amino acids critical for SLPI's antiprotease activity (L72K, M73G, L74G) could reduce cell death, infarct size, cardiac injury markers, and improved cardiac functions, by attenuating intracellular signalling molecules involved in cell death, including p38 MAPK, Bax, and Caspase-8.

In a myocardial ischaemia/reperfusion injury, the level of protease enzymes activity level increased for both intracellular and extracellular cardiac cells in order to maintain homeostasis of cellular structure by degrading of damaged proteins (12). Normally, protease enzymes play a crucial role in producing and degrading proteins in order to recycle dysfunctional and damaged proteins in the cell (43). The myocardial injury during ischaemia contributes to the accumulation of activated neutrophils in the injury area. The accumulation of activated neutrophil leads to tissue injury as result of releasing of cytotoxic cytokines including inflammatory cytokines and proteases enzymes which eventually results in cell death (335). Moreover, after reperfusion, these were attributed to more activated reactive oxygen species (ROS), inflammatory cytokines, and the accumulation of neutrophils (22, 324). Increasing in ROS and inflammatory cytokines levels triggered the recruitment and infiltration of neutrophils and mast cells, which released serine proteases including cathepsin, calpain and matrix metalloproteinase (MMP), which are key mediators involved in cardiovascular diseases progression (336). The post-MI patients showed an increased level of protease enzyme in serum which is related to growth factors levels which is connected to the fibrosis development (337, 338). Therefore, the protease enzyme that plays a crucial role in the development and progression of cardiovascular diseases. In addition, an imbalance of protease activity as well as protease inhibitors in the cell were contributed to cardiac cell injury and the changing the structure of cardiac tissue as cardiac remodelling or cardiac hypertrophy (12). Therefore, the inhibition of serine protease enzymes has therapeutic potential and applications to prevent the progression of cardiac remodelling or heart failure after myocardial infarction.

The protease enzyme inhibitory strategy has been shown to provide a therapeutic potential to reduce cardiac cell death, injury, and remodelling in several

study models (339-342), which also include the study from our study on SLPI. SLPI have been proved to reduce cardiac cell death in ischaemia/reperfusion injury, which is believed to reduced reactive oxygen species, attenuated cell stress signalling, and enhanced cell survival pathway, which subsequently attenuated the apoptotic regulatory pathways (22, 23, 307, 308, 310-312). This evidence suggests that inhibition of protease activity could be a major contributing mechanism explaining the therapeutic potential of SLPI. However, the direct effect of SLPI itself, regardless of its anti-protease activity, has also been demonstrated.

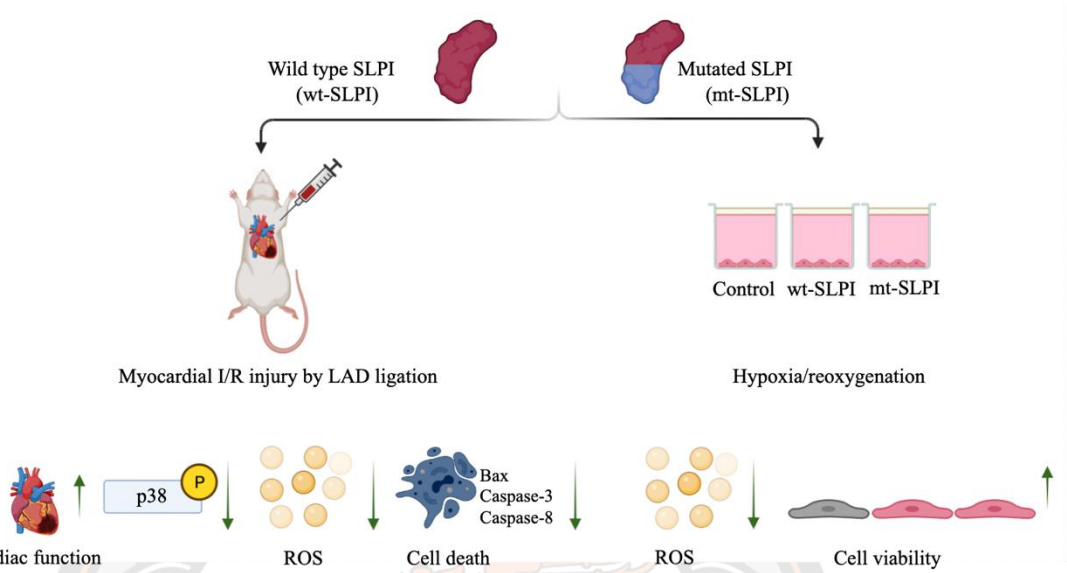
It is well known that the central role of the protease inhibitory effect of SLPI located at Leu^{72}-Met^{73} residues (343). However, the mechanism of the direct effect of SLPI as well as intracellular signal transduction downstream of SLPI stimulation is still unclear. Mutation of the critical amino acids that govern SLPI anti-protease activity have been implemented and the results were interpreted in such a way that losing the ability to inhibit serine protease could not affect the beneficial effect of SLPI, particularly on anti-HIV infection and anti-inflammation (319, 325). Yet, anti-protease activity deficient SLPI has never been investigated with cardiovascular diseases, particularly myocardial ischaemia/reperfusion injury. Our study demonstrates, as first evidence on the cardiovascular model, that anti-protease activity deficient SLPI treatment in post-ischaemic condition could reduce infarct size, cardiac biomarkers, inflammatory cytokines and improve cardiac function (Figure 34).

Anti-protease deficient SLPI (L72K, M73G, L74G) has been generated and studies have been conducted with an immunological study model (319). The potential mechanism of anti-protease deficient SLPI was intracellular interaction and competing binding of SLPI to p65 which prevented the activation of NF-κB and resulted in reduced of TNF-α and IL-8 production (344). In addition, SLPI binding partners have been identified, association of SLPI with a calcium dependent phospholipid binding membrane Annexin A2 (AnxA2) enhanced AnxA2 phosphorylation (227), which consequently mediates Akt-survival pathway activation (345). Besides, SLPI can bind to phospholipid cellular receptors-scramblases 1 and 4 which resulted in inhibition of HIV-1 infection (177). Scramblases can bind to onzin, the c-Myc transcription factor. The association of scramblases and onzin could interact with Akt and Mdm2, inhibit p53, and lead to inhibiting cell apoptosis (346).

The information indicates that SLPI could have a direct effect signalling without anti-protease activity by binding to molecules involved in the survival pathway. Although the anti-protease independent mechanism of SLPI against cell death has been reported, the mechanism related to cardioprotection still requires elucidation.

Serine proteases constitute the most numerous groups of proteases, accounting for 40% of them, and they are prevalent in many physiological functions, both normal and disease-related functions (347-349). Inhibition of serine proteases also interferes with physiological homeostasis. The anti-protease independent effect of SLPI in this current study in the context of myocardial I/R injury, which could point out the ***"Direct Effect"*** of SLPI on cardioprotection. Implementation of anti-protease deficient recombinant-SLPI could be more clinically beneficial in terms of providing cardioprotection without interfering with basal serine protease activity. Therefore, further investigation of anti-protease deficient recombinant-SLPI in a more clinical related study should be performed since our findings convinced that it would provide very useful information. Since the half-life of SLPI after administration is short, approximately 60 min in the circulation (200), any strategies to prolong the half-life of SLPI, increase its stability, or to offer a more efficient delivery method required investigation. A previous study using chimeric protein of SLPI-Cementoin fusion protein found that it could successfully enhance the biological activity of SLPI (350). Therefore, chimeric of anti-protease deficient SLPI-Cementoin has an interesting potential to enhance the physiological activity of anti-protease deficient recombinant-SLPI.

Our study demonstrates, for the ***first time***, that rhSLPI treatment after coronary artery occlusion and treatment of anti-protease deficient recombinant SLPI



protein in an *in vivo* rat model. rhSLPI and anti-protease deficient recombinant SLPI protein could reduce cardiac cell injury in an *in vitro* hypoxia/reoxygenation, reduced infarct size, and improve the cardiac function in rats subjected to an in vivo myocardial I/R injury. Our findings suggested that SLPI could provide cardioprotection without anti-protease activity.

Figure 34 A schematic diagram of the major findings in this study. Both type of wild type and mutant (anti-protease deficient) SLPI could improve cell viability, reduce infarct size and improve cardiac function. These could associate with attenuation of p38 MAPK activation, intracellular ROS production, and activation of apoptotic regulatory signalling proteins, Bax, Caspase-3 and Caspase-8.

Limitation of this study

There are some limitations in the current study that need to be addressed. The major source of protease secretion in myocardial I/R injury is from inflammatory cells in the heart There are some limitations in the current study that need to be addressed. The major after reperfusion, particularly neutrophiles (3). In this study, cardiac tissue inflammatory source of protease secretion in myocardial I/R injury is from inflammatory cells in the cytokines were measured from a ventricular tissue

homogenate, which is believed to heart after reperfusion, particularly neutrophiles (3). In this study, cardiac tissue inflammation contains a certain number of leukocytes in the cardiac tissue. Therefore, the inflammatory cytokines were measured from a ventricular tissue homogenate, which is believed cytokines level shown in this study is actually from both ventricular tissue and residence leukocytes. The immunohistochemistry on the ventricular tissue section to determine or quantify infiltrated inflammatory cells needs to be investigated. Furthermore, assessment of protease activity, particularly cathepsin G and chymase, from ventricular tissue extract should also be performed by an in vitro cleavage of a fluorescent peptide substrate (324). It is noteworthy that although our current study showed that treatment of rhSLPI could reduce p38 MAPK phosphorylation and activate Akt/ERK phosphorylation, this evidence could not provide a direct proof that SLPI protects the heart from I/R injury through those signalling pathways. The findings from this study, as well as the previous reports, could only suggest the association of those cell survival pathways with the protective effect of SLPI. Therefore, abolishing the protection, by using inhibitors of both pathways, could confirm the involvement of cell survival pathways on cardioprotection. In part of anti-protease deficiency SLPI, the consequences of I/R injury included both cell injury in addition to cellular inflammation and immune responses, which are known to be involved in the disease progression (68). The inflammatory cytokines level, leukocytes infiltration, and response of protease enzymes which secreted from infiltrate leukocytes, in the presence of mutant SLPI treatment, should be investigated. As discussed previously concerning the direct effect of SLPI in association with the binding partners, such as Annexin A2, scramblases 1 and 4. This information will provide more mechanistic insight about the cardioprotective effect of SLPI.

Design of future studies

It is also suggested that, for further investigation, the association between SLPI and those candidates' binding partners in myocardial I/R injury are challenging. Moreover, the effect of long-term treatment of rhSLPI in myocardial infarction and post-myocardial infarction cardiac remodeling and heart failure progression should be assessed to provide the useful data before moving forward to clinical trials.

The SLPI mechanism was not clearly investigated. However, previous studies showed the binding protein partner of SLPI, Annexin A2, which was upregulated along with SLPI expression in human smoking mucosa samples from squamous cell carcinoma of the head and neck (HNSCC) (351). The binding between Annexin A2 and SLPI prevented human papillomavirus (HPV) infection by competitively binding with Annexin A2 against HPV. Furthermore, an HIV-1 infection study found that SLPI binding to Annexin A2 heterotetramer (A2t) on the macrophage surface, including A2 and S100A10, impairs productive HIV-1 infection. However, the mechanism has not been determined. Annexin A2 antibody inhibited HIV-1 infection, whereas A2t inhibitor did not (352). Other SLPI binding partner binding protein was phospholipid scramblases 1 and 4 which were cellular receptors for regulating inner and outer plasma membrane movement (177). SLPI could disrupt the binding between CD4 and scramblase receptors because the results showed SLPI binding to scramblase receptor 1 on the same region with CD4 of HIV-1 that can modulating the association between scramblase receptors and CD4 (177).

Previous studies showed that SLPI was discovered to have a rapid clearance rate in both human plasma and urine. SLPI is normally eliminated through glomerular filtration, then reabsorption and degradation in tubular cells, and in the 2-6 h interval, the estimated half-life of SLPI in plasma is 120 minutes (200). Besides, SLPI is inactivated by enzyme that is secreted from respiratory tract such as cathepsin (353). Thus, transportation of SLPI to target organ is new challenge. Therefore, any strategies to improve the stability and extend the half-life of SLPI in system, could provide greater therapeutic benefit. Several advantages of nanoparticles for peptide drug carriers have been listed, for example reducing the enzymatic digestion and aggregation of peptide drugs, increasing the transmembrane absorption. Therefore, fabrication of nanoparticle encapsulated rhSLPI and its efficiency for I/R injury need to be investigated.

Reference

REFERENCES

1. Celermajer DS, Chow CK, Marijon E, Anstey NM, Woo KS. Cardiovascular disease in the developing world: prevalences, patterns, and the potential of early disease detection. J Am Coll Cardiol. 2012;60(14):1207-16.
2. Müller AL, Hryshko LV, Dhalla NS. Extracellular and intracellular proteases in cardiac dysfunction due to ischemia–reperfusion injury. International Journal of Cardiology. 2013;164(1):39-47.
3. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. Cardiovasc Res. 2002;53(1):31-47.
4. Zahler S, Massoudy P, Hartl H, Hähnel C, Meisner H, Becker BF. Acute cardiac inflammatory responses to postischemic reperfusion during cardiopulmonary bypass. Cardiovasc Res. 1999;41(3):722-30.
5. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. Int Rev Cell Mol Biol. 2012;298:229-317.
6. Chohan PK, Singh RB, Dhalla NS, Netticadan T. L-arginine administration recovers sarcoplasmic reticulum function in ischemic reperfused hearts by preventing calpain activation. Cardiovasc Res. 2006;69(1):152-63.
7. Cuervo AM, Wong ES, Martinez-Vicente M. Protein degradation, aggregation, and misfolding. Mov Disord. 2010;25 Suppl 1:S49-54.
8. Powell SR, Wang P, Katzeff H, Shringarpure R, Teoh C, Khaliulin I, et al. Oxidized and ubiquitinated proteins may predict recovery of postischemic cardiac function: essential role of the proteasome. Antioxid Redox Signal. 2005;7(5-6):538-46.
9. Singh RB, Chohan PK, Dhalla NS, Netticadan T. The sarcoplasmic reticulum proteins are targets for calpain action in the ischemic-reperfused heart. J Mol Cell Cardiol. 2004;37(1):101-10.
10. Singh RB, Dandekar SP, Elimban V, Gupta SK, Dhalla NS. Role of proteases in the pathophysiology of cardiac disease. Molecular and cellular biochemistry. 2004;263(1-2):241-56.
11. Willis MS, Schisler JC, Portbury AL, Patterson C. Build it up-Tear it down: protein quality control in the cardiac sarcomere. Cardiovasc Res. 2009;81(3):439-48.
12. Müller AL, Dhalla NS. Role of various proteases in cardiac remodeling and progression of heart failure. Heart Fail Rev. 2012;17(3):395-409.
13. Nieuwenhuijs-Moeke GJ, Pischke SE, Berger SP, Sanders JSF, Pol RA, Struys MMRF, et al. Ischemia and Reperfusion Injury in Kidney Transplantation: Relevant Mechanisms in Injury and Repair. Journal of Clinical Medicine. 2020;9(1):253.
14. Urata H, Kinoshita A, Misono KS, Bumpus FM, Husain A. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. The Journal of biological chemistry. 1990;265(36):22348-57.
15. Amberger A, Schneeberger S, Hernegger G, Brandacher G, Obrist P, Lackner P, et al. Gene expression profiling of prolonged cold ischemia and reperfusion in murine heart transplants. Transplantation. 2002;74(10):1441-9.
16. Schneeberger S, Brandacher G, Mark W, Amberger A, Margreiter R. Protease Inhibitors as a Potential Target in Modulation of Postischemic Inflammation. Drug News Perspect. 2002;15(9):568-74.
17. Schneeberger S, Hautz T, Wahl SM, Brandacher G, Sucher R, Steinmassl O, et al. The effect of secretory leukocyte protease inhibitor (SLPI) on ischemia/reperfusion

injury in cardiac transplantation. Am J Transplant. 2008;8(4):773-82.
18. Walter M, Plotnick M, Schechter NM. Inhibition of Human Mast Cell Chymase by Secretory Leukocyte Proteinase Inhibitor: Enhancement of the Interaction by Heparin. Archives of Biochemistry and Biophysics. 1996;327(1):81-8.
19. Wright CD, Havill AM, Middleton SC, Kashem MA, Lee PA, Dripps DJ, et al. Secretory leukocyte protease inhibitor prevents allergen-induced pulmonary responses in animal models of asthma. The Journal of pharmacology and experimental therapeutics. 1999;289(2):1007-14.
20. Majchrzak-Gorecka M, Majewski P, Grygier B, Murzyn K, Cichy J. Secretory leukocyte protease inhibitor (SLPI), a multifunctional protein in the host defense response. Cytokine Growth Factor Rev. 2016;28:79-93.
21. Miller KW, Evans RJ, Eisenberg SP, Thompson RC. Secretory leukocyte protease inhibitor binding to mRNA and DNA as a possible cause of toxicity to Escherichia coli. Journal of Bacteriology. 1989;171(4):2166.
22. Prompunt E, Sanit J, Barrère-Lemaire S, Nargeot J, Noordali H, Madhani M, et al. The cardioprotective effects of secretory leukocyte protease inhibitor against myocardial ischemia/reperfusion injury. Experimental and therapeutic medicine. 2018;15(6):5231-42.
23. Prompunt E, Nernpermpisooth N, Sanit J, Kumphune S. Overexpression and pre-treatment of recombinant human Secretory Leukocyte Protease Inhibitor (rhSLPI) reduces an in vitro ischemia/reperfusion injury in rat cardiac myoblast (H9c2) cell. Biomolecular concepts. 2018;9(1):17-32.
24. McGarry N, Greene CM, McElvaney NG, Weldon S, Taggart CC. The Ability of Secretory Leukocyte Protease Inhibitor to Inhibit Apoptosis in Monocytes Is Independent of Its Antiprotease Activity. J Immunol Res. 2015;2015:507315.
25. Yang J, Zhu J, Sun D, Ding A. Suppression of macrophage responses to bacterial lipopolysaccharide (LPS) by secretory leukocyte protease inhibitor (SLPI) is independent of its anti-protease function. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2005;1745(3):310-7.
26. (WHO) WHO. Cardiovascular diseases (CVDs): 11 June 2021; 2021 [Available from: https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).
27. Federation WH. Cardiovascular Disease The world's number 1 killer World Heart Federation; 2022 [Available from: https://world-heart-federation.org/resource/cardiovascular-disease-infographic/.
28. Virani SS, Alonso A, Aparicio HJ, Benjamin EJ, Bittencourt MS, Callaway CW, et al. Heart Disease and Stroke Statistics—2021 Update. Circulation. 2021;143(8):e254-e743.
29. Buja LM. Myocardial ischemia and reperfusion injury. Cardiovasc Pathol. 2005;14(4):170-5.
30. Nakamura M. Angiography is the gold standard and objective evidence of myocardial ischemia is mandatory if lesion severity is questionable. - Indication of PCI for angiographically significant coronary artery stenosis without objective evidence of myocardial ischemia (Pro). Circ J. 2011;75(1):204-10; discussion 17.
31. Xia Z, Chen Y, Fan Q, Xue M. Oxidative stress-mediated reperfusion injury: mechanism and therapies. Oxidative medicine and cellular longevity. 2014;2014:373081-.

32. de Vries DK, Kortekaas KA, Tsikas D, Wijermars LGM, van Noorden CJF, Suchy M-T, et al. Oxidative damage in clinical ischemia/reperfusion injury: a reappraisal. Antioxidants & redox signaling. 2013;19(6):535-45.
33. Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. The Journal of clinical investigation. 2013;123(1):92-100.
34. Glogar DH. [Definition and significance of the area at risk in myocardial infarct and the ischemic border zone in acute myocardial infarct]. Acta medica Austriaca Supplement. 1986;36:1-40.
35. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium–apoptosis link. Nature Reviews Molecular Cell Biology. 2003;4:552.
36. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nature reviews Molecular cell biology. 2010;11(9):621-32.
37. Sanada S, Komuro I, Kitakaze M. Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures. American journal of physiology Heart and circulatory physiology. 2011;301(5):H1723-41.
38. Muñoz-Pinedo C, Guío-Carrión A, Goldstein JC, Fitzgerald P, Newmeyer DD, Green DR. Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(31):11573-8.
39. Kim HE, Du F, Fang M, Wang X. Formation of apoptosome is initiated by cytochrome c-induced dATP hydrolysis and subsequent nucleotide exchange on Apaf-1. Proc Natl Acad Sci U S A. 2005;102(49):17545-50.
40. Malladi S, Challa-Malladi M, Fearnhead HO, Bratton SB. The Apaf-1*procaspase-9 apoptosome complex functions as a proteolytic-based molecular timer. The EMBO journal. 2009;28(13):1916-25.
41. Moldoveanu T, Liu Q, Tocilj A, Watson M, Shore G, Gehring K. The X-ray structure of a BAK homodimer reveals an inhibitory zinc binding site. Molecular cell. 2006;24(5):677-88.
42. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. The New England journal of medicine. 2007;357(11):1121-35.
43. Hua Y, Nair S. Proteases in cardiometabolic diseases: Pathophysiology, molecular mechanisms and clinical applications. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2015;1852(2):195-208.
44. Yamamoto F, Yamamoto H, Yoshida S, Ichikawa H, Takahashi A, Tanaka K, et al. The effects of several pharmacologic agents upon postischemic recovery. Cardiovascular drugs and therapy. 1991;5 Suppl 2:301-8.
45. Cox TR, Erler JT. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis Model Mech. 2011;4(2):165-78.
46. Antoniak S, Pawlinski R, Mackman N. Protease-activated receptors and myocardial infarction. IUBMB Life. 2011;63(6):383-9.
47. Hernando V, Inserte J, Sartorio CL, Parra VM, Poncelas-Nozal M, Garcia-Dorado D. Calpain translocation and activation as pharmacological targets during myocardial ischemia/reperfusion. J Mol Cell Cardiol. 2010;49(2):271-9.
48. Mani SK, Balasubramanian S, Zavadzkas JA, Jeffords LB, Rivers WT, Zile MR,

et al. Calpain inhibition preserves myocardial structure and function following myocardial infarction. American journal of physiology Heart and circulatory physiology. 2009;297(5):H1744-51.
49. Inserte J, Barba I, Hernando V, Garcia-Dorado D. Delayed recovery of intracellular acidosis during reperfusion prevents calpain activation and determines protection in postconditioned myocardium. Cardiovascular Research. 2009;81(1):116-22.
50. Sorimachi H, Imajoh-Ohmi S, Emori Y, Kawasaki H, Ohno S, Minami Y, et al. Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and mu-types. Specific expression of the mRNA in skeletal muscle. The Journal of biological chemistry. 1989;264(33):20106-11.
51. Schwartz SM, Duffy JY, Pearl J, Goins S, Wagner CJ, Nelson DP. Glucocorticoids preserve calpastatin and troponin I during cardiopulmonary bypass in immature pigs. Pediatric research. 2003;54(1):91-7.
52. Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, et al. Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics. 2012;1824(1):68-88.
53. Ganote CE, Seabra-Gomes R, Nayler WG, Jennings RB. Irreversible myocardial injury in anoxic perfused rat hearts. The American journal of pathology. 1975;80(3):419-50.
54. Cheng XW, Murohara T, Kuzuya M, Izawa H, Sasaki T, Obata K, et al. Superoxide-dependent cathepsin activation is associated with hypertensive myocardial remodeling and represents a target for angiotensin II type 1 receptor blocker treatment. Am J Pathol. 2008;173(2):358-69.
55. Felbor U, Dreier L, Bryant RA, Ploegh HL, Olsen BR, Mothes W. Secreted cathepsin L generates endostatin from collagen XVIII. The EMBO journal. 2000;19(6):1187-94.
56. Everts V, Korper W, Hoeben KA, Jansen ID, Bromme D, Cleutjens KB, et al. Osteoclastic bone degradation and the role of different cysteine proteinases and matrix metalloproteinases: differences between calvaria and long bone. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2006;21(9):1399-408.
57. Ali MA, Schulz R. Activation of MMP-2 as a key event in oxidative stress injury to the heart. Frontiers in bioscience (Landmark edition). 2009;14(2):699-716.
58. Cavdar Z, Ural C, Celik A, Arslan S, Terzioglu G, Ozbal S, et al. Protective effects of taurine against renal ischemia/reperfusion injury in rats by inhibition of gelatinases, MMP-2 and MMP-9, and p38 mitogen-activated protein kinase signaling. Biotech Histochem. 2017;92(7):524-35.
59. Sawicki G. Intracellular regulation of matrix metalloproteinase-2 activity: new strategies in treatment and protection of heart subjected to oxidative stress. Scientifica. 2013;2013:130451-.
60. Lalu MM, Pasini E, Schulze CJ, Ferrari-Vivaldi M, Ferrari-Vivaldi G, Bachetti T, et al. Ischaemia-reperfusion injury activates matrix metalloproteinases in the human heart. European heart journal. 2005;26(1):27-35.
61. Tiwari M, Hemalatha T, Ganesan K, Nayeem M, Murali Manohar B, Balachandran C, et al. Myocardial ischemia and reperfusion injury in rats: lysosomal hydrolases and matrix metalloproteinases mediated cellular damage. Molecular and

cellular biochemistry. 2008;312(1-2):81-91.
62. Kukacka J, Prusa R, Kotaska K, Pelouch V. Matrix metalloproteinases and their function in myocardium. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia. 2005;149(2):225-36.
63. Stewart James A, Wei C-C, Brower Gregory L, Rynders Patricia E, Hankes Gerald H, Dillon AR, et al. Cardiac mast cell- and chymase-mediated matrix metalloproteinase activity and left ventricular remodeling in mitral regurgitation in the dog. Journal of Molecular and Cellular Cardiology. 2003;35(3):311-9.
64. Zhang H, Wu J, Dong H, Khan Shaukat A, Chu M-L, Tsuda T. Fibulin-2 deficiency attenuates angiotensin II-induced cardiac hypertrophy by reducing transforming growth factor-β signalling. Clinical Science. 2014;126(4):275-88.
65. Foronjy RF, Sun J, Lemaitre V, D'Armiento JM. Transgenic Expression of Matrix Metalloproteinase-1 Inhibits Myocardial Fibrosis and Prevents the Transition to Heart Failure in a Pressure Overload Mouse Model. Hypertension Research. 2008;31:725.
66. Matsusaka H, Ide T, Matsushima S, Ikeuchi M, Kubota T, Sunagawa K, et al. Targeted Deletion of Matrix Metalloproteinase 2 Ameliorates Myocardial Remodeling in Mice With Chronic Pressure Overload. Hypertension. 2006;47(4):711-7.
67. Pluijmert NJ, Atsma DE, Quax PHA. Post-ischemic Myocardial Inflammatory Response: A Complex and Dynamic Process Susceptible to Immunomodulatory Therapies. Frontiers in Cardiovascular Medicine. 2021;8.
68. Liu D, Zeng X, Li X, Mehta JL, Wang X. Role of NLRP3 inflammasome in the pathogenesis of cardiovascular diseases. Basic Res Cardiol. 2018;113(1):5.
69. Assadiasl S, Mousavi MJ, Amirzargar A. Toll-Like Receptor 4 in Renal Transplant. Exp Clin Transplant. 2018;16(3):245-52.
70. Rusai K, Sollinger D, Baumann M, Wagner B, Strobl M, Schmaderer C, et al. Toll-like receptors 2 and 4 in renal ischemia/reperfusion injury. Pediatr Nephrol. 2010;25(5):853-60.
71. Salvadori M, Rosso G, Bertoni E. Update on ischemia-reperfusion injury in kidney transplantation: Pathogenesis and treatment. World J Transplant. 2015;5(2):52-67.
72. Stancovski I, Baltimore D. NF-kappaB activation: the I kappaB kinase revealed? Cell. 1997;91(3):299-302.
73. Yang M-q, Ma Y-y, Ding J, Li J-y. The role of mast cells in ischemia and reperfusion injury. Inflammation Research. 2014;63(11):899-905.
74. Sack MN, Smith RM, Opie LH. Tumor necrosis factor in myocardial hypertrophy and ischaemia — an anti-apoptotic perspective. Cardiovascular Research. 2000;45(3):688-95.
75. Ong S-B, Hernández-Reséndiz S, Crespo-Avilan GE, Mukhametshina RT, Kwek X-Y, Cabrera-Fuentes HA, et al. Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic opportunities. Pharmacol Ther. 2018;186:73-87.
76. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Ischemia/Reperfusion. Comprehensive Physiology. 2016;7(1):113-70.
77. Heller RA, Schena M, Chai A, Shalon D, Bedilion T, Gilmore J, et al. Discovery and analysis of inflammatory disease-related genes using cDNA microarrays. Proceedings of the National Academy of Sciences of the United States of America.

1997;94(6):2150-5.
78. Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits. Biochimica et biophysica acta. 2005;1754(1-2):253-62.
79. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nature reviews Immunology. 2003;3(9):745-56.
80. Weiss SJ. Tissue destruction by neutrophils. The New England journal of medicine. 1989;320(6):365-76.
81. Chiba Y, Morioka K, Muraoka R, Ihaya A, Kimura T, Uesaka T, et al. Effects of depletion of leukocytes and platelets on cardiac dysfunction after cardiopulmonary bypass. Ann Thorac Surg. 1998;65(1):107-13; discussion 13-4.
82. Sharony R, Yu P-J, Park J, Galloway AC, Mignatti P, Pintucci G. Protein targets of inflammatory serine proteases and cardiovascular disease. Journal of Inflammation. 2010;7(1):45.
83. Ueno M, Moriyama Y, Toda R, Yotsumoto G, Yamamoto H, Fukumoto Y, et al. Effect of a neutrophil elastase inhibitor (ONO-5046 Na) on ischemia/reperfusion injury using the left-sided heterotopic canine heart transplantation model. The Journal of Heart and Lung Transplantation. 2001;20(8):889-96.
84. Yamazaki T, Ooshima H, Usui A, Watanabe T, Yasuura K. Protective effects of ONO-5046·Na, a specific neutrophil elastase inhibitor, on postperfusion lung injury. The Annals of Thoracic Surgery. 1999;68(6):2141-6.
85. Tomizawa N, Ohwada S, Ohya T, Kawashima Y, Takeyoshi I, Morishita Y. The effect of neutrophil elastase inhibitor in hepatectomy with ischemia in dogs. J Surg Res. 1999;81(2):230-7.
86. Kambe M, Bessho R, Fujii M, Ochi M, Shimizu K. Sivelestat reduces myocardial ischemia and reperfusion injury in rat hearts even when administered after onset of myocardial ischemia☆. Interactive CardioVascular and Thoracic Surgery. 2009;8(6):629-34.
87. Krzywonos-Zawadzka A, Franczak A, Olejnik A, Radomski M, Gilmer JF, Sawicki G, et al. Cardioprotective effect of MMP-2-inhibitor-NO-donor hybrid against ischaemia/reperfusion injury. J Cell Mol Med. 2019;23(4):2836-48.
88. Krzywonos-Zawadzka A, Franczak A, Sawicki G, Bil-Lula I. Mixture of MMP-2, MLC, and NOS Inhibitors Affects NO Metabolism and Protects Heart from Cardiac I/R Injury. Cardiol Res Pract. 2020;2020:1561478.
89. Hooshdaran B, Kolpakov M, Rafiq K, Guo X, Kiani M, Sabri A. Targeting inflammatory serine proteases for cardiac repair after Ischemia Reperfusion injury (1080.10). The FASEB Journal. 2014;28.
90. Kolpakov M, Hooshdaran B, Guo X, Rafiq K, Vlasenko L, Bashkirova Y, et al. Inhibition of inflammatory serine proteases promotes vascularization and enhances cardiac repair after myocardial infarction. Journal of Molecular and Cellular Cardiology. 2018;124:92-3.
91. Pruefer D, Buerke U, Khalil M, Dahm M, Darius H, Oelert H, et al. Cardioprotective effects of the serine protease inhibitor aprotinin after regional ischemia and reperfusion on the beating heart. J Thorac Cardiovasc Surg. 2002;124(5):942-9.
92. Aga Q, Nair A, Hourani W, Parasuraman S, Kk M, Sreeharsha N. Therapeutic Potential of Chymase Inhibitors in Cardiovascular Diseases: An Overview. Indian Journal of Pharmaceutical Education and Research. 2020;54:509-16.

93. Bot I, Bot M, van Heiningen SH, van Santbrink PJ, Lankhuizen IM, Hartman P, et al. Mast cell chymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in ApoE-/- mice. Cardiovasc Res. 2011;89(1):244-52.
94. Jin D, Takai S, Yamada M, Sakaguchi M, Kamoshita K, Ishida K, et al. Impact of chymase inhibitor on cardiac function and survival after myocardial infarction. Cardiovasc Res. 2003;60(2):413-20.
95. Sukenaga Y, Kamoshita K, Takai S, Miyazaki M. Development of the chymase inhibitor as an anti-tissue-remodeling drug: myocardial infarction and some other possibilities. Jpn J Pharmacol. 2002;90(3):218-22.
96. Soga Y, Takai S, Koyama T, Okamoto Y, Ikeda T, Nishimura K, et al. Attenuation of adhesion formation after cardiac surgery with a chymase inhibitor in a hamster model. The Journal of Thoracic and Cardiovascular Surgery. 2004;127(1):72-8.
97. Arooj M, Sakkiah S, Cao GP, Kim S, Arulalapperumal V, Lee KW. Finding off-targets, biological pathways, and target diseases for chymase inhibitors via structure-based systems biology approach. Proteins. 2015;83(7):1209-24.
98. Pejler G. Novel Insight into the in vivo Function of Mast Cell Chymase: Lessons from Knockouts and Inhibitors. Journal of Innate Immunity. 2020;12(5):357-72.
99. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516.
100. Martin JL, Gruszczyk AV, Beach TE, Murphy MP, Saeb-Parsy K. Mitochondrial mechanisms and therapeutics in ischaemia reperfusion injury. Pediatr Nephrol. 2019;34(7):1167-74.
101. Rossello X, Yellon DM. The RISK pathway and beyond. Basic Res Cardiol. 2018;113(1):2.
102. Del Re DP, Amgalan D, Linkermann A, Liu Q, Kitsis RN. Fundamental Mechanisms of Regulated Cell Death and Implications for Heart Disease. Physiological Reviews. 2019;99(4):1765-817.
103. Green DR, Ferguson TA. The role of Fas ligand in immune privilege. Nature Reviews Molecular Cell Biology. 2001;2(12):917-24.
104. Jeremias I, Kupatt C, Martin-Villalba A, Habazettl H, Schenkel J, Boekstegers P, et al. Involvement of CD95/Apo1/Fas in Cell Death After Myocardial Ischemia. Circulation. 2000;102(8):915-20.
105. Bardwell L, Thorner J. A conserved motif at the amino termini of MEKs might mediate high-affinity interaction with the cognate MAPKs. Trends Biochem Sci. 1996;21(10):373-4.
106. Weston CR, Lambright DG, Davis RJ. Signal transduction. MAP kinase signaling specificity. Science (New York, NY). 2002;296(5577):2345-7.
107. Cuenda A, Rousseau S. p38 MAP-Kinases pathway regulation, function and role in human diseases. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2007;1773(8):1358-75.
108. Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC. An osmosensing signal transduction pathway in yeast. Science (New York, NY). 1993;259(5102):1760-3.
109. Goedert M, Cuenda A, Craxton M, Jakes R, Cohen P. Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. The EMBO journal. 1997;16(12):3563-71.

110. Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, et al. MAP kinases. Chem Rev. 2001;101(8):2449-76.
111. Wang Z, Harkins PC, Ulevitch RJ, Han J, Cobb MH, Goldsmith EJ. The structure of mitogen-activated protein kinase p38 at 2.1-A resolution. Proc Natl Acad Sci U S A. 1997;94(6):2327-32.
112. Gräb J, Rybniker J. The Expanding Role of p38 Mitogen-Activated Protein Kinase in Programmed Host Cell Death. Microbiol Insights. 2019;12:1178636119864594.
113. Ono K, Han J. The p38 signal transduction pathway: activation and function. Cellular signalling. 2000;12(1):1-13.
114. Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, et al. Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. Circulation research. 1996;79(2):162-73.
115. Chang CI, Xu BE, Akella R, Cobb MH, Goldsmith EJ. Crystal structures of MAP kinase p38 complexed to the docking sites on its nuclear substrate MEF2A and activator MKK3b. Molecular cell. 2002;9(6):1241-9.
116. Meier R, Rouse J, Cuenda A, Nebreda AR, Cohen P. Cellular stresses and cytokines activate multiple mitogen-activated-protein kinase kinase homologues in PC12 and KB cells. European journal of biochemistry. 1996;236(3):796-805.
117. Deacon K, Mistry P, Chernoff J, Blank JL, Patel R. p38 Mitogen-activated protein kinase mediates cell death and p21-activated kinase mediates cell survival during chemotherapeutic drug-induced mitotic arrest. Molecular biology of the cell. 2003;14(5):2071-87.
118. Rousseau S, Houle F, Landry J, Huot J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Oncogene. 1997;15(18):2169-77.
119. Cheng HL, Feldman EL. Bidirectional regulation of p38 kinase and c-Jun N-terminal protein kinase by insulin-like growth factor-I. The Journal of biological chemistry. 1998;273(23):14560-5.
120. Foltz IN, Lee JC, Young PR, Schrader JW. Hemopoietic Growth Factors with the Exception of Interleukin-4 Activate the p38 Mitogen-activated Protein Kinase Pathway. Journal of Biological Chemistry. 1997;272(6):3296-301.
121. Kyriakis JM, Avruch J. pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. The Journal of biological chemistry. 1990;265(28):17355-63.
122. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev. 2001;81(2):807-69.
123. Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, et al. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature. 1997;389(6653):865-70.
124. Cui J, Zhang M, Zhang Y-q, Xu Z-h. JNK pathway: diseases and therapeutic potential. Acta Pharmacologica Sinica. 2007;28:601.
125. Dhanasekaran DN, Reddy EP. JNK-signaling: A multiplexing hub in programmed cell death. Genes Cancer. 2017;8(9-10):682-94.

126. Yamamoto K, Ichijo H, Korsmeyer SJ. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Molecular and cellular biology. 1999;19(12):8469-78.
127. Chen Q, Xu T, Li D, Pan D, Wu P, Luo Y, et al. JNK/PI3K/Akt signaling pathway is involved in myocardial ischemia/reperfusion injury in diabetic rats: effects of salvianolic acid A intervention. Am J Transl Res. 2016;8(6):2534-48.
128. Xu W, Zhang L, Zhang Y, Zhang K, Wu Y, Jin D. TRAF1 Exacerbates Myocardial Ischemia Reperfusion Injury via ASK1-JNK/p38 Signaling. J Am Heart Assoc. 2019;8(21):e012575.
129. Schulman D, Latchman DS, Yellon DM. Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. American Journal of Physiology-Heart and Circulatory Physiology. 2002;283(4):H1481-H8.
130. Nagoshi T, Matsui T, Aoyama T, Leri A, Anversa P, Li L, et al. PI3K rescues the detrimental effects of chronic Akt activation in the heart during ischemia/reperfusion injury. The Journal of Clinical Investigation. 2005;115(8):2128-38.
131. Mensah K, Mocanu MM, Yellon DM. Failure to protect the myocardium against ischemia/reperfusion injury after chronic atorvastatin treatment is recaptured by acute atorvastatin treatment: A potential role for phosphatase and tensin homolog deleted on chromosome ten? Journal of the American College of Cardiology. 2005;45(8):1287-91.
132. Hausenloy DJ, Duchen MR, Yellon DM. Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia–reperfusion injury. Cardiovascular Research. 2003;60(3):617-25.
133. Coffer PJ, Woodgett JR. Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. European journal of biochemistry. 1991;201(2):475-81.
134. Jones PF, Jakubowicz T, Pitossi FJ, Maurer F, Hemmings BA. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. Proc Natl Acad Sci U S A. 1991;88(10):4171-5.
135. Bellacosa A, Testa JR, Staal SP, Tsichlis PN. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science (New York, NY). 1991;254(5029):274-7.
136. Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord JM. Serine/threonine protein kinases and apoptosis. Exp Cell Res. 2000;256(1):34-41.
137. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res. 2004;61(3):448-60.
138. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997;91(2):231-41.
139. Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc Natl Acad Sci U S A. 2001;98(17):9666-70.
140. Yamaguchi H, Wang HG. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. Oncogene. 2001;20(53):7779-86.
141. Kennedy SG, Kandel ES, Cross TK, Hay N. Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. Molecular and

cellular biology. 1999;19(8):5800-10.
142. Hallberg B, Rayter SI, Downward J. Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. The Journal of biological chemistry. 1994;269(6):3913-6.
143. Yndestad A, Sandanger Ø, Jong WMC, Aukrust P, Zuurbier CJ. Response to letter from Toldo et al. on "NLRP3 inflammasome activation during myocardial ischemia reperfusion is cardioprotective". Biochemical and Biophysical Research Communications. 2016;474(2):328-9.
144. Yang M, Chen J, Zhao J, Meng M. Etanercept Attenuates Myocardial Ischemia/Reperfusion Injury by Decreasing Inflammation and Oxidative Stress. PLOS ONE. 2014;9(9):e108024.
145. Duan J, Yang Y, Liu H, Dou P-C, Tan S-Y. Osthole ameliorates acute myocardial infarction in rats by decreasing the expression of inflammatory-related cytokines, diminishing MMP-2 expression and activating p-ERK. Int J Mol Med. 2016;37(1):207-16.
146. Zhang J, Song J, Xu J, Chen X, Yin P, Lv X, et al. ERK1/2-Egr-1 Signaling Pathway-Mediated Protective Effects of Electroacupuncture in a Mouse Model of Myocardial Ischemia-Reperfusion. Evid Based Complement Alternat Med. 2014;2014:253075.
147. Ferrari R, Guardigli G, Mele D, Percoco GF, Ceconi C, Curello S. Oxidative stress during myocardial ischaemia and heart failure. Current pharmaceutical design. 2004;10(14):1699-711.
148. Imlay JA. Pathways of oxidative damage. Annu Rev Microbiol. 2003;57:395-418.
149. Selle T, Renger I, Labidi S, Bultmann I, Hilfiker-Kleiner D. Reviewing peripartum cardiomyopathy: current state of knowledge. Future Cardiol. 2009;5(2):175-89.
150. Halliwell B. Biochemistry of oxidative stress. Biochem Soc Trans. 2007;35(Pt 5):1147-50.
151. Yao H, Rahman I. Current concepts on oxidative/carbonyl stress, inflammation and epigenetics in pathogenesis of chronic obstructive pulmonary disease. Toxicol Appl Pharmacol. 2011;254(2):72-85.
152. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, et al. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 1990;186:464-78.
153. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med. 2006;10(2):389-406.
154. Dimitrijevic ZM, Salinger Martinovic SS, Nikolic VN, Cvetkovic TP. Protein Carbonyl Content Is a Predictive Biomarker of Eccentric Left Ventricular Hypertrophy in Hemodialysis Patients. Diagnostics (Basel). 2019;9(4).
155. Curtis JM, Hahn WS, Long EK, Burrill JS, Arriaga EA, Bernlohr DA. Protein carbonylation and metabolic control systems. Trends in Endocrinology & Metabolism. 2012;23(8):399-406.
156. Park AM, Suzuki YJ. Effects of intermittent hypoxia on oxidative stress-induced myocardial damage in mice. J Appl Physiol (1985). 2007;102(5):1806-14.
157. Ichihara S, Suzuki Y, Chang J, Kuzuya K, Inoue C, Kitamura Y, et al.

Involvement of oxidative modification of proteins related to ATP synthesis in the left ventricles of hamsters with cardiomyopathy. Scientific Reports. 2017;7(1):9243.
158. Gaze DC. Ischemia modified albumin: a novel biomarker for the detection of cardiac ischemia. Drug Metab Pharmacokinet. 2009;24(4):333-41.
159. Mothes E, Faller P. Evidence that the principal CoII-binding site in human serum albumin is not at the N-terminus: implication on the albumin cobalt binding test for detecting myocardial ischemia. Biochemistry. 2007;46(8):2267-74.
160. Sinha MK, Vazquez JM, Calvino R, Gaze DC, Collinson PO, Kaski JC. Effects of balloon occlusion during percutaneous coronary intervention on circulating Ischemia Modified Albumin and transmyocardial lactate extraction. Heart. 2006;92(12):1852.
161. Wu AH, Morris DL, Fletcher DR, Apple FS, Christenson RH, Painter PC. Analysis of the Albumin Cobalt Binding (ACB) test as an adjunct to cardiac troponin I for the early detection of acute myocardial infarction. Cardiovasc Toxicol. 2001;1(2):147-51.
162. Wei Z, Liu G, Jia R, Zhang W, Li L, Zhang Y, et al. Targeting secretory leukocyte protease inhibitor (SLPI) inhibits colorectal cancer cell growth, migration and invasion via downregulation of AKT. PeerJ. 2020;8:e9400.
163. Grütter MG, Fendrich G, Huber R, Bode W. The 2.5 A X-ray crystal structure of the acid-stable proteinase inhibitor from human mucous secretions analysed in its complex with bovine alpha-chymotrypsin. The EMBO journal. 1988;7(2):345-51.
164. Clauss A, Lilja H, Lundwall A. A locus on human chromosome 20 contains several genes expressing protease inhibitor domains with homology to whey acidic protein. Biochem J. 2002;368(Pt 1):233-42.
165. Eisenberg SP, Hale KK, Heimdal P, Thompson RC. Location of the protease-inhibitory region of secretory leukocyte protease inhibitor. The Journal of biological chemistry. 1990;265(14):7976-81.
166. Kramps JA, van Twisk C, Appelhans H, Meckelein B, Nikiforov T, Dijkman JH. Proteinase inhibitory activities of antileukoprotease are represented by its second COOH-terminal domain. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology. 1990;1038(2):178-85.
167. Baranger K, Zani ML, Labas V, Dallet-Choisy S, Moreau T. Secretory leukocyte protease inhibitor (SLPI) is, like its homologue trappin-2 (pre-elafin), a transglutaminase substrate. PLoS One. 2011;6(6):e20976.
168. Forteza RM, Ahmed A, Lee T, Abraham WM. Secretory leukocyte protease inhibitor, but not alpha-1 protease inhibitor, blocks tryptase-induced bronchoconstriction. Pulm Pharmacol Ther. 2001;14(2):107-10.
169. Li Z, Moy A, Gomez SR, Franz AH, Lin-Cereghino J, Lin-Cereghino GP. An improved method for enhanced production and biological activity of human secretory leukocyte protease inhibitor (SLPI) in Pichia pastoris. Biochemical and Biophysical Research Communications. 2010;402(3):519-24.
170. Zhang Y, DeWitt DL, McNeely TB, Wahl SM, Wahl LM. Secretory leukocyte protease inhibitor suppresses the production of monocyte prostaglandin H synthase-2, prostaglandin E2, and matrix metalloproteinases. J Clin Invest. 1997;99(5):894-900.
171. Wen J, Nikitakis NG, Chaisuparat R, Greenwell-Wild T, Gliozzi M, Jin W, et al. Secretory leukocyte protease inhibitor (SLPI) expression and tumor invasion in oral squamous cell carcinoma. Am J Pathol. 2011;178(6):2866-78.
172. Hiemstra PS, Maassen RJ, Stolk J, Heinzel-Wieland R, Steffens GJ, Dijkman

JH. Antibacterial activity of antileukoprotease. Infect Immun. 1996;64(11):4520-4.
173. Wiedow O, Harder J, Bartels J, Streit V, Christophers E. Antileukoprotease in human skin: an antibiotic peptide constitutively produced by keratinocytes. Biochem Biophys Res Commun. 1998;248(3):904-9.
174. Tarhini M, Pizzoccaro A, Benlyamani I, Rebaud C, Greige-Gerges H, Fessi H, et al. Human serum albumin nanoparticles as nanovector carriers for proteins: Application to the antibacterial proteins "neutrophil elastase" and "secretory leukocyte protease inhibitor". Int J Pharm. 2020;579:119150.
175. Nugteren S, Samsom JN. Secretory Leukocyte Protease Inhibitor (SLPI) in mucosal tissues: Protects against inflammation, but promotes cancer. Cytokine Growth Factor Rev. 2021;59:22-35.
176. McNeely TB, Dealy M, Dripps DJ, Orenstein JM, Eisenberg SP, Wahl SM. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. J Clin Invest. 1995;96(1):456-64.
177. Py B, Basmaciogullari S, Bouchet J, Zarka M, Moura IC, Benhamou M, et al. The Phospholipid Scramblases 1 and 4 Are Cellular Receptors for the Secretory Leukocyte Protease Inhibitor and Interact with CD4 at the Plasma Membrane. PLOS ONE. 2009;4(3):e5006.
178. Tseng C-C, Tseng C-P. Identification of a novel secretory leukocyte protease inhibitor-binding protein involved in membrane phospholipid movement. FEBS Letters. 2000;475(3):232-6.
179. BÖHm B, Aigner T, Kinne R, Burkhardt H. The serine-protease inhibitor of cartilage matrix is not a chondrocytic gene product. European journal of biochemistry. 1992;207(2):773-9.
180. Maruyama M, Hay JG, Yoshimura K, Chu CS, Crystal RG. Modulation of secretory leukoprotease inhibitor gene expression in human bronchial epithelial cells by phorbol ester. The Journal of Clinical Investigation. 1994;94(1):368-75.
181. Mihaila A, Tremblay GM. Human Alveolar Macrophages Express Elafin and Secretory Leukocyte Protease Inhibitor. Zeitschrift für Naturforschung C. 2001;56(3-4):291-7.
182. Ohlsson M, Rosengren M, Tegner H, Ohlsson K. Quantification of Granulocyte Elastase Inhibitors in Human Mixed Saliva and in Pure Parotid Secretion. 1983;364(2):1323-8.
183. Jin FY, Nathan C, Radzioch D, Ding A. Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. Cell. 1997;88(3):417-26.
184. Menckeberg CL, Hol J, Simons-Oosterhuis Y, Raatgeep HC, de Ruiter LF, Lindenbergh-Kortleve DJ, et al. Human buccal epithelium acquires microbial hyporesponsiveness at birth, a role for secretory leukocyte protease inhibitor. Gut. 2015;64(6):884.
185. Vos JB, van Sterkenburg MA, Rabe KF, Schalkwijk J, Hiemstra PS, Datson NA. Transcriptional response of bronchial epithelial cells to Pseudomonas aeruginosa: identification of early mediators of host defense. Physiol Genomics. 2005;21(3):324-36.
186. McCartney-Francis N, Jin W, Belkaid Y, McGrady G, Wahl SM. Aberrant host defense against Leishmania major in the absence of SLPI. J Leukoc Biol. 2014;96(5):917-29.
187. Wahl SM. Secretory leukocyte protease inhibitor-an endogenous antimicrobial

agent. AIDS. 2001;15.
188. Svensson D, Aidoukovitch A, Anders E, Jönsson D, Nebel D, Nilsson BO. Secretory leukocyte protease inhibitor regulates human periodontal ligament cell production of pro-inflammatory cytokines. Inflamm Res. 2017;66(9):823-31.
189. Si-Tahar M, Merlin D, Sitaraman S, Madara JL. Constitutive and regulated secretion of secretory leukocyte proteinase inhibitor by human intestinal epithelial cells. Gastroenterology. 2000;118(6):1061-71.
190. King AE, Fleming DC, Critchley HO, Kelly RW. Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells. Mol Hum Reprod. 2002;8(4):341-9.
191. Tehrani FA, Modaresifar K, Azizian S, Niknejad H. Induction of antimicrobial peptides secretion by IL-1β enhances human amniotic membrane for regenerative medicine. Sci Rep. 2017;7(1):17022.
192. Odaka C, Mizuochi T, Yang J, Ding A. Murine macrophages produce secretory leukocyte protease inhibitor during clearance of apoptotic cells: implications for resolution of the inflammatory response. J Immunol. 2003;171(3):1507-14.
193. Jin F, Nathan CF, Radzioch D, Ding A. Lipopolysaccharide-related stimuli induce expression of the secretory leukocyte protease inhibitor, a macrophage-derived lipopolysaccharide inhibitor. Infect Immun. 1998;66(6):2447-52.
194. Yang J, Zhu J, Sun D, Ding A. Suppression of macrophage responses to bacterial lipopolysaccharide (LPS) by secretory leukocyte protease inhibitor (SLPI) is independent of its anti-protease function. Biochim Biophys Acta. 2005;1745(3):310-7.
195. Klimenkova O, Ellerbeck W, Klimiankou M, Ünalan M, Kandabarau S, Gigina A, et al. A lack of secretory leukocyte protease inhibitor (SLPI) causes defects in granulocytic differentiation. Blood. 2014;123(8):1239-49.
196. Kramps JA, van Twisk C, Klasen EC, Dijkman JH. Interactions among stimulated human polymorphonuclear leucocytes, released elastase and bronchial antileucoprotease. Clin Sci (Lond). 1988;75(1):53-62.
197. Vandooren J, Goeminne P, Boon L, Ugarte-Berzal E, Rybakin V, Proost P, et al. Neutrophils and Activated Macrophages Control Mucosal Immunity by Proteolytic Cleavage of Antileukoproteinase. Front Immunol. 2018;9:1154.
198. Belkowski SM, Masucci J, Mahan A, Kervinen J, Olson M, de Garavilla L, et al. Cleaved SLPI, a novel biomarker of chymase activity. Biol Chem. 2008;389(9):1219-24.
199. Walter M, Plotnick M, Schechter NM. Inhibition of human mast cell chymase by secretory leukocyte proteinase inhibitor: enhancement of the interaction by heparin. Arch Biochem Biophys. 1996;327(1):81-8.
200. Bergenfeldt M, Bjork P, Ohlsson K. The elimination of secretory leukocyte protease inhibitor (SLPI) after intravenous injection in dog and man. Scand J Clin Lab Invest. 1990;50(7):729-37.
201. Fernie-King BA, Seilly DJ, Davies A, Lachmann PJ. Streptococcal inhibitor of complement inhibits two additional components of the mucosal innate immune system: secretory leukocyte proteinase inhibitor and lysozyme. Infect Immun. 2002;70(9):4908-16.
202. O. Wiedow JH, J. Bartels, V. Streit, E. Christophers, . Antileukoprotease in human skin: an antibiotic peptide constitutively produced by keratinocytes, Biochem. . Biophys Res Commun. 1998;248:904–9.

203. P.S. Hiemstra RJM, J. Stolk, R. Heinzel-Wieland, G.J. Steffens, J., H. Dijkman. Antibacterial activity of antileukoprotease. Infect Immun 1996;64:4520–4.
204. Verma C, Seebah S, Low SM, Zhou L, Liu SP, Li J, et al. Defensins: antimicrobial peptides for therapeutic development. Biotechnol J. 2007;2(11):1353-9.
205. J.F. Tomee PSH, R. Heinzel-Wieland, H.F. Kauffman, . Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi, J. Infect. Dis. 176 (1997) 740–747. J Infect Dis. 1997;176:740–7.
206. Miller KW, Evans RJ, Eisenberg SP, Thompson RC. Secretory leukocyte protease inhibitor binding to mRNA and DNA as a possible cause of toxicity to Escherichia coli. Journal of Bacteriology. 1989;171(4):2166-72.
207. Hiemstra PS, Maassen RJ, Stolk J, Heinzel-Wieland R, Steffens GJ, Dijkman JH. Antibacterial activity of antileukoprotease. Infect Immun. 1996;64(11):4520-4.
208. Wiedow O, Harder J, Bartels J, Streit V, Christophers E. Antileukoprotease in Human Skin: An Antibiotic Peptide Constitutively Produced by Keratinocytes. Biochemical and Biophysical Research Communications. 1998;248(3):904-9.
209. Si-Tahar M, Merlin D, Sitaraman S, Madara JL. Constitutive and regulated secretion of secretory leukocyte proteinase inhibitor by human intestinal epithelial cells. Gastroenterology. 2000;118(6):1061-71.
210. Fernie-King BA, Seilly DJ, Davies A, Lachmann PJ. Streptococcal inhibitor of complement inhibits two additional components of the mucosal innate immune system: secretory leukocyte proteinase inhibitor and lysozyme. Infect Immun. 2002;70(9):4908-16.
211. Gomez SA, Argüelles CL, Guerrieri D, Tateosian NL, Amiano NO, Slimovich R, et al. Secretory leukocyte protease inhibitor: a secreted pattern recognition receptor for mycobacteria. Am J Respir Crit Care Med. 2009;179(3):247-53.
212. Cooper MD, Roberts MH, Barauskas OL, Jarvis GA. Secretory Leukocyte Protease Inhibitor Binds to Neisseria gonorrhoeae Outer Membrane Opacity Protein and is Bactericidal. American Journal of Reproductive Immunology. 2012;68(2):116-27.
213. Grobmyer SR, Barie PS, Nathan CF, Fuortes M, Lin E, Lowry SF, et al. Secretory leukocyte protease inhibitor, an inhibitor of neutrophil activation, is elevated in serum in human sepsis and experimental endotoxemia. Critical Care Medicine. 2000;28(5).
214. Nakamura A, Mori Y, Hagiwara K, Suzuki T, Sakakibara T, Kikuchi T, et al. Increased susceptibility to LPS-induced endotoxin shock in secretory leukoprotease inhibitor (SLPI)-deficient mice. J Exp Med. 2003;197(5):669-74.
215. Nishimura J, Saiga H, Sato S, Okuyama M, Kayama H, Kuwata H, et al. Potent Antimycobacterial Activity of Mouse Secretory Leukocyte Protease Inhibitor. The Journal of Immunology. 2008;180(6):4032-9.
216. Jin F, C. Nathan, D. Radzioch, and A. Ding. Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. Cell. 1997;88:417–26.
217. Taggart CC, Greene CM, McElvaney NG, O'Neill S. Secretory leucoprotease inhibitor prevents lipopolysaccharide-induced IkappaBalpha degradation without affecting phosphorylation or ubiquitination. The Journal of biological chemistry. 2002;277(37):33648-53.
218. Taggart CC, Cryan SA, Weldon S, Gibbons A, Greene CM, Kelly E, et al. Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and

inhibits p65 binding. J Exp Med. 2005;202(12):1659-68.
219. Liu L, Ma Z, Sun WW, Cao JP. Upregulated Expression of Secretory Leukocyte Protease Inhibitor in Lung by Inhalation of High Concentration of Sulfur Dioxide. Chin Med J (Engl). 2018;131(16):2005-7.
220. Jin F-y, Nathan C, Radzioch D, Ding A. Secretory Leukocyte Protease Inhibitor: A Macrophage Product Induced by and Antagonistic to Bacterial Lipopolysaccharide. Cell. 1997;88(3):417-26.
221. Xu W, He B, Chiu A, Chadburn A, Shan M, Buldys M, et al. Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. Nature Immunology. 2007;8(3):294-303.
222. Tomee JF, Hiemstra PS, Heinzel-Wieland R, Kauffman HF. Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi. J Infect Dis. 1997;176(3):740-7.
223. Farquhar C, VanCott TC, Mbori-Ngacha DA, Horani L, Bosire RK, Kreiss JK, et al. Salivary secretory leukocyte protease inhibitor is associated with reduced transmission of human immunodeficiency virus type 1 through breast milk. J Infect Dis. 2002;186(8):1173-6.
224. McNeely TB, Shugars DC, Rosendahl M, Tucker C, Eisenberg SP, Wahl SM. Inhibition of Human Immunodeficiency Virus Type 1 Infectivity by Secretory Leukocyte Protease Inhibitor Occurs Prior to Viral Reverse Transcription. Blood. 1997;90(3):1141-9.
225. Raynor CM, Wright JF, Waisman DM, Pryzdial EL. Annexin II enhances cytomegalovirus binding and fusion to phospholipid membranes. Biochemistry. 1999;38(16):5089-95.
226. Malhotra R, Ward M, Bright H, Priest R, Foster MR, Hurle M, et al. Isolation and characterisation of potential respiratory syncytial virus receptor(s) on epithelial cells. Microbes Infect. 2003;5(2):123-33.
227. Ma G, Greenwell-Wild T, Lei K, Jin W, Swisher J, Hardegen N, et al. Secretory leukocyte protease inhibitor binds to annexin II, a cofactor for macrophage HIV-1 infection. J Exp Med. 2004;200(10):1337-46.
228. Moriyama A, Shimoya K, Ogata I, Kimura T, Nakamura T, Wada H, et al. Secretory leukocyte protease inhibitor (SLPI) concentrations in cervical mucus of women with normal menstrual cycle. Mol Hum Reprod. 1999;5(7):656-61.
229. Abe T, Kobayashi N, Yoshimura K, Trapnell BC, Kim H, Hubbard RC, et al. Expression of the secretory leukoprotease inhibitor gene in epithelial cells. J Clin Invest. 1991;87(6):2207-15.
230. Wang X, Li X, Xu L, Zhan Y, Yaish-Ohad S, Erhardt JA, et al. Up-regulation of secretory leukocyte protease inhibitor (SLPI) in the brain after ischemic stroke: adenoviral expression of SLPI protects brain from ischemic injury. Mol Pharmacol. 2003;64(4):833-40.
231. Ghasemlou N, Bouhy D, Yang J, López-Vales R, Haber M, Thuraisingam T, et al. Beneficial effects of secretory leukocyte protease inhibitor after spinal cord injury. Brain. 2010;133(Pt 1):126-38.
232. Hentschel J, Fischer N, Janhsen WK, Markert UR, Lehmann T, Sonnemann J, et al. Protease-antiprotease imbalances differ between Cystic Fibrosis patients' upper and lower airway secretions. J Cyst Fibros. 2015;14(3):324-33.
233. Müller U, Hentschel J, Janhsen WK, Hünniger K, Hipler UC, Sonnemann J, et

al. Changes of Proteases, Antiproteases, and Pathogens in Cystic Fibrosis Patients' Upper and Lower Airways after IV-Antibiotic Therapy. Mediators Inflamm. 2015;2015:626530.
234. Habgood AN, Tatler AL, Porte J, Wahl SM, Laurent GJ, John AE, et al. Secretory leukocyte protease inhibitor gene deletion alters bleomycin-induced lung injury, but not development of pulmonary fibrosis. Lab Invest. 2016;96(6):623-31.
235. Velarde MC, Parisek SI, Eason RR, Simmen FA, Simmen RC. The secretory leukocyte protease inhibitor gene is a target of epidermal growth factor receptor action in endometrial epithelial cells. J Endocrinol. 2005;184(1):141-51.
236. Meyer M, Bauer RN, Letang BD, Brighton L, Thompson E, Simmen RCM, et al. Regulation and activity of secretory leukoprotease inhibitor (SLPI) is altered in smokers. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2014;306(3):L269-L76.
237. Raundhal M, Morse C, Khare A, Oriss TB, Milosevic J, Trudeau J, et al. High IFN-γ and low SLPI mark severe asthma in mice and humans. J Clin Invest. 2015;125(8):3037-50.
238. Israel E, Reddel HK. Severe and Difficult-to-Treat Asthma in Adults. New England Journal of Medicine. 2017;377(10):965-76.
239. Facciolongo N, Bonacini M, Galeone C, Ruggiero P, Menzella F, Ghidoni G, et al. Bronchial thermoplasty in severe asthma: a real-world study on efficacy and gene profiling. Allergy, Asthma & Clinical Immunology. 2022;18(1):39.
240. Ano S, Kikuchi N, Matsuyama M, Nakajima M, Kondo Y, Masuda M, et al. Transcriptome genetic differences between responders and non-responders before bronchial thermoplasty. J Asthma. 2021:1-11.
241. Owen J, Yost N, Berghella V, Thom E, Swain M, Dildy GA, 3rd, et al. Mid-trimester endovaginal sonography in women at high risk for spontaneous preterm birth. Jama. 2001;286(11):1340-8.
242. Choi SR, Hong SS, Kim J, Lee KY. Neutrophil elastase in cervical fluid in women with short cervical length. Taiwan J Obstet Gynecol. 2018;57(3):407-10.
243. Samejima T, Nagamatsu T, Akiba N, Fujii T, Sayama S, Kawana K, et al. Secretory leukocyte protease inhibitor and progranulin as possible regulators of cervical remodeling in pregnancy. J Reprod Immunol. 2021;143:103241.
244. Kanninen TT, Sisti G, Ramer I, Goldschlag D, Witkin SS, Spandorfer SD. Predictive biomarkers of preterm delivery in women with ongoing IVF pregnancies. J Reprod Immunol. 2015;112:58-62.
245. Wyatt KA, Filby CE, Davies-Tuck ML, Suke SG, Evans J, Gargett CE. Menstrual fluid endometrial stem/progenitor cell and supernatant protein content: cyclical variation and indicative range. Hum Reprod. 2021;36(8):2215-29.
246. Madendag IC, Sahin ME, Madendag Y, Sahin E, Karakukcu C. Evaluation of the levels of secretory leukocyte protease inhibitor in the cervical mucus of women with unexplained infertility. J Obstet Gynaecol Res. 2020;46(7):1128-32.
247. Marth CD, Firestone SM, Hanlon D, Glenton LY, Browning GF, Young ND, et al. Innate immune genes in persistent mating-induced endometritis in horses. Reprod Fertil Dev. 2018;30(3):533-45.
248. Kim HS, Ha K-S, Kwon HC, Lee SJ, Kim C-H, Cheon Y-P. Enhancing the developmental competence of the early embryo using secretory leukocyte peptidase inhibitor. Differentiation. 2016;92(1):24-34.

249. Karvellas CJ, Pink F, McPhail M, Cross T, Auzinger G, Bernal W, et al. Predictors of bacteraemia and mortality in patients with acute liver failure. Intensive Care Medicine. 2009;35(8):1390-6.
250. Antoniades CG, Khamri W, Abeles RD, Taams LS, Triantafyllou E, Possamai LA, et al. Secretory leukocyte protease inhibitor: a pivotal mediator of anti-inflammatory responses in acetaminophen-induced acute liver failure. Hepatology. 2014;59(4):1564-76.
251. Li S, Wang S, Murugan R, Al-Khafaji A, Lebovitz DJ, Souter M, et al. Donor biomarkers as predictors of organ use and recipient survival after neurologically deceased donor organ transplantation. J Crit Care. 2018;48:42-7.
252. Averdunk L, Fitzner C, Levkovich T, Leaf DE, Sobotta M, Vieten J, et al. Secretory Leukocyte Protease Inhibitor (SLPI)-A Novel Predictive Biomarker of Acute Kidney Injury after Cardiac Surgery: A Prospective Observational Study. J Clin Med. 2019;8(11).
253. Wilflingseder J, Sunzenauer J, Toronyi E, Heinzel A, Kainz A, Mayer B, et al. Molecular Pathogenesis of Post-Transplant Acute Kidney Injury: Assessment of Whole-Genome mRNA and MiRNA Profiles. PLOS ONE. 2014;9(8):e104164.
254. Ambrosi NG, Caro FY, Osella F, Alvarez LD, Sánchez F, Toniolo F, et al. SLPI in the perfusion solution helps to identify graft quality in kidney transplants. Biomark Med. 2019;13(11):895-906.
255. Averdunk L, Rückbeil MV, Zarbock A, Martin L, Marx G, Jalaie H, et al. SLPI - a Biomarker of Acute Kidney Injury after Open and Endovascular Thoracoabdominal Aortic Aneurysm (TAAA) Repair. Scientific Reports. 2020;10(1):3453.
256. Ibrahim M, Vece G, Mehew J, Johnson R, Forsythe J, Klassen D, et al. An international comparison of deceased donor kidney utilization: What can the United States and the United Kingdom learn from each other? American Journal of Transplantation. 2020;20(5):1309-22.
257. Yang B, Sylvius N, Luo J, Yang C, Da Z, Crotty C, et al. Identifying Biomarkers from Transcriptomic Signatures in Renal Allograft Biopsies Using Deceased and Living Donors. Frontiers in Immunology. 2021;12.
258. Wu Y, Chen W, Zhang Y, Liu A, Yang C, Wang H, et al. Potent Therapy and Transcriptional Profile of Combined Erythropoietin-Derived Peptide Cyclic Helix B Surface Peptide and Caspase-3 siRNA against Kidney Ischemia/Reperfusion Injury in Mice. The Journal of pharmacology and experimental therapeutics. 2020;375(1):92-103.
259. Brooks DE, Ollivier FJ. Matrix metalloproteinase inhibition in corneal ulceration. Vet Clin North Am Small Anim Pract. 2004;34(3):611-22.
260. Reviglio VE, Sambuelli RH, Olmedo A, Falco M, Echenique J, O'Brien TP, et al. Secretory leukocyte protease inhibitor is an inducible antimicrobial peptide expressed in Staphylococcus aureus endophthalmitis. Mediators Inflamm. 2007;2007:93857.
261. Reviglio VE, Grenat A, Pegoraro F, Sambuelli RH, Rana T, Kuo IC. Secretory Leukoprotease Inhibitor: A Native Antimicrobial Protein in the Innate Immune Response in a Rat Model of S. aureus Keratitis. J Ophthalmol. 2009;2009:259393.
262. Martel-Pelletier J, Barr AJ, Cicuttini FM, Conaghan PG, Cooper C, Goldring MB, et al. Osteoarthritis. Nature Reviews Disease Primers. 2016;2(1):16072.
263. Oikonomopoulou K, Diamandis EP, Hollenberg MD, Chandran V. Proteinases and their receptors in inflammatory arthritis: an overview. Nature Reviews

Rheumatology. 2018;14(3):170-80.
264. Mehana E-SE, Khafaga AF, El-Blehi SS. The role of matrix metalloproteinases in osteoarthritis pathogenesis: An updated review. Life Sciences. 2019;234:116786.
265. Kim HE, Shin Y, Jung IJ, Yang JI, Chun CH, Kim HA, et al. Overexpression of secretory leukocyte peptidase inhibitor (SLPI) does not modulate experimental osteoarthritis but may be a biomarker for the disease. Osteoarthritis Cartilage. 2021;29(4):558-67.
266. Timur UT, Jahr H, Anderson J, Green DC, Emans PJ, Smagul A, et al. Identification of tissue-dependent proteins in knee OA synovial fluid. Osteoarthritis Cartilage. 2021;29(1):124-33.
267. Narayanan K, Ramachandran A, Hao J, He G, Park KW, Cho M, et al. Dual functional roles of dentin matrix protein 1. Implications in biomineralization and gene transcription by activation of intracellular Ca2+ store. The Journal of biological chemistry. 2003;278(19):17500-8.
268. Jeong SJ, Wang G, Choi BD, Hwang YH, Kim BH, Ko YM, et al. Secretory Leukocyte Protease Inhibitor (SLPI) Increases Focal Adhesion in MC3T3 Osteoblast on Titanium Surface. J Nanosci Nanotechnol. 2015;15(1):200-4.
269. Choi BD, Lee SY, Jeong SJ, Lim DS, Cha HJ, Chung WG, et al. Secretory leukocyte protease inhibitor promotes differentiation and mineralization of MC3T3-E1 preosteoblasts on a titanium surface. Mol Med Rep. 2016;14(2):1241-6.
270. Jeong JO, Wang G, Jeong SJ, Choi BD, Lee HY, Jeong MJ. Function of Secretory Leukocyte Protease Inhibitor (SLPI) in Odontoblast During Mouse Tooth Development. J Nanosci Nanotechnol. 2015;15(1):120-4.
271. Song D, Yang S, Tan T, Wang R, Ma Z, Wang Y, et al. ODAM promotes junctional epithelium-related gene expression via activation of WNT1 signaling pathway in an ameloblast-like cell line ALC. J Periodontal Res. 2021;56(3):482-91.
272. Lee SY, Moon JS, Yang DW, Yoo HI, Jung JY, Kim OS, et al. SLPI in periodontal Ligament is not sleepy during biophysical force-induced tooth movement. J Clin Periodontol. 2021;48(4):528-40.
273. Tirali RE, Yalçınkaya Erdemci Z, Çehreli SB. Oral findings and clinical implications of patients with congenital neutropenia: a literature review. Turk J Pediatr. 2013;55(3):241-5.
274. Sriwattanapong K, Sa-Ard-Iam N, Boonprakong L, Subbalekha K, Trachoo V, Suratannon N, et al. Reduced ELANE and SLPI expression compromises dental pulp cell activity. Cell Prolif. 2021;54(11):e13132.
275. Liu G, Yang J, Zhao Y, Wang Z, Xing B, Wang L, et al. Expression of secretory leukocyte protease inhibitor detected by immunohistochemistry correlating with prognosis and metastasis in colorectal cancer. World Journal of Surgical Oncology. 2014;12(1):369.
276. Zhang W, Yao JL, Dong SC, Hou FQ, Shi HP. SLPI knockdown induced pancreatic ductal adenocarcinoma cells proliferation and invasion. Cancer Cell Int. 2015;15:37.
277. Zuo J, Zhang C, Ren C, Pang D, Li Y, Xie X, et al. Secretory leukocyte protease inhibitor is a proliferation and survival factor for pancreatic cancer cells. Clin Transl Oncol. 2015;17(4):314-21.
278. Du XY, Liu X, Wang ZJ, Wang YY. SLPI promotes the gastric cancer growth and metastasis by regulating the expression of P53, Bcl-2 and Caspase-8. Eur Rev Med

Pharmacol Sci. 2017;21(7):1495-501.
279. Xie W, Zhang H, Qin S, Zhang J, Fan X, Yin Y, et al. The expression and clinical significance of secretory leukocyte proteinase inhibitor (SLPI) in mammary carcinoma using bioinformatics analysis. Gene. 2019;720:144088.
280. Kozin SV, Maimon N, Wang R, Gupta N, Munn L, Jain RK, et al. Secretory leukocyte protease inhibitor (SLPI) as a potential target for inhibiting metastasis of triple-negative breast cancers. Oncotarget. 2017;8(65).
281. Sayers KT, Brooks AD, Sayers TJ, Chertov O. Increased secretory leukocyte protease inhibitor (SLPI) production by highly metastatic mouse breast cancer cells. PLoS One. 2014;9(8):e104223.
282. Rosso M, Lapyckyj L, Amiano N, Besso MJ, Sánchez M, Chuluyan E, et al. Secretory Leukocyte Protease Inhibitor (SLPI) expression downregulates E-cadherin, induces β-catenin re-localisation and triggers apoptosis-related events in breast cancer cells. Biology of the Cell. 2014;106(9):308-22.
283. Vanharanta S, Massagué J. Origins of metastatic traits. Cancer Cell. 2013;24(4):410-21.
284. Wagenblast E, Soto M, Gutiérrez-Ángel S, Hartl CA, Gable AL, Maceli AR, et al. A model of breast cancer heterogeneity reveals vascular mimicry as a driver of metastasis. Nature. 2015;520(7547):358-62.
285. Stępień T, Brożyna M, Kuzdak K, Motylewska E, Komorowski J, Stępień H, et al. Elevated Concentrations of SERPINE2/Protease Nexin-1 and Secretory Leukocyte Protease Inhibitor in the Serum of Patients with Papillary Thyroid Cancer. Disease Markers. 2017;2017:4962137.
286. Reyes I, Reyes N, Suriano R, Iacob C, Suslina N, Policastro A, et al. Gene expression profiling identifies potential molecular markers of papillary thyroid carcinoma. Cancer Biomark. 2019;24(1):71-83.
287. Quabius ES, Möller P, Haag J, Pfannenschmidt S, Hedderich J, Görögh T, et al. The role of the antileukoprotease SLPI in smoking-induced human papillomavirus-independent head and neck squamous cell carcinomas. Int J Cancer. 2014;134(6):1323-34.
288. Pierce Campbell CM, Giuliano AR, Torres BN, O'Keefe MT, Ingles DJ, Anderson RL, et al. Salivary secretory leukocyte protease inhibitor (SLPI) and head and neck cancer: The Cancer Prevention Study II Nutrition Cohort. Oral Oncology. 2016;55:1-5.
289. Noorlag R, van der Groep P, Leusink FKJ, van Hooff SR, Frank MH, Willems SM, et al. Nodal metastasis and survival in oral cancer: Association with protein expression of SLPI, not with LCN2, TACSTD2, or THBS2. Head & Neck. 2015;37(8):1130-6.
290. Dickey BL, Sirak B, Martin-Gomez L, Reich RR, Abrahamsen M, Isaacs-Soriano K, et al. Oral secretory leukocyte protease inhibitor (SLPI): Associations with oropharyngeal cancer and treatment outcome. PLoS One. 2021;16(7):e0254161.
291. Mikami Y, Fukushima A, Komiyama Y, Iwase T, Tsuda H, Higuchi Y, et al. Human uterus myoma and gene expression profiling: A novel in vitro model for studying secretory leukocyte protease inhibitor-mediated tumor invasion. Cancer Letters. 2016;379(1):84-93.
292. Mizutani Y, Omagari D, Hayatsu M, Nameta M, Komiyama K, Mikami Y, et al. SLPI facilitates cell migration by regulating lamellipodia/ruffles and desmosomes, in

which Galectin4 plays an important role. Cell Adh Migr. 2020;14(1):195-203.
293. Takamura T, Suguro H, Mikami Y, Iwase T, Komiyama Y, Kuyama K, et al. Comparison of gene expression profiles of gingival carcinoma Ca9-22 cells and colorectal adenocarcinoma HT-29 cells to identify potentially important mediators of SLPI-induced cell migration. J Oral Sci. 2017;59(2):279-87.
294. Yang Y, Rhodus NL, Ondrey FG, Wuertz BRK, Chen X, Zhu Y, et al. Quantitative Proteomic Analysis of Oral Brush Biopsies Identifies Secretory Leukocyte Protease Inhibitor as a Promising, Mechanism-Based Oral Cancer Biomarker. PLOS ONE. 2014;9(4):e95389.
295. Wang X, Jin Y, Li Y-X, Yang Y. Secretory leukocyte peptidase inhibitor expression and apoptosis effect in oral leukoplakia and oral squamous cell carcinoma. Oncol Rep. 2018;39(4):1793-804.
296. Yang Z, Chen J, Xie H, Liu T, Chen Y, Ma Z, et al. Androgen receptor suppresses prostate cancer metastasis but promotes bladder cancer metastasis via differentially altering miRNA525-5p/SLPI-mediated vasculogenic mimicry formation. Cancer Lett. 2020;473:118-29.
297. Zheng D, Gui B, Gray KP, Tinay I, Rafiei S, Huang Q, et al. Secretory leukocyte protease inhibitor is a survival and proliferation factor for castration-resistant prostate cancer. Oncogene. 2016;35(36):4807-15.
298. Xu J, Lv S, Meng W, Zuo F. LCN2 Mediated by IL-17 Affects the Proliferation, Migration, Invasion and Cell Cycle of Gastric Cancer Cells by Targeting SLPI. Cancer Manag Res. 2020;12:12841-9.
299. Meehan R, Lewis JD, Bird AP. Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. Nucleic Acids Research. 1992;20(19):5085-92.
300. Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet. 1999;23(2):185-8.
301. Liu C, Wang J, Wei Y, Zhang W, Geng M, Yuan Y, et al. Fat-Specific Knockout of Mecp2 Upregulates Slpi to Reduce Obesity by Enhancing Browning. Diabetes. 2019;69(1):35-47.
302. Liu X, Li S, Wang L, Zhang W, Wang Y, Gui L, et al. The Effect of FATP1 on Adipocyte Differentiation in Qinchuan Beef Cattle. Animals. 2021;11(10):2789.
303. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovasc Res. 2004;61(3):481-97.
304. Liu R, Chen L, Wu W, Chen H, Zhang S. Neutrophil serine proteases and their endogenous inhibitors in coronary artery ectasia patients. Anatol J Cardiol. 2016;16(1):23-8.
305. Mendez IJ, Manemann SM, Bell EJ, Larson NB, Decker PA, Guerrero MA, et al. Adhesion pathway proteins and risk of atrial fibrillation in the Multi-Ethnic Study of Atherosclerosis. BMC Cardiovascular Disorders. 2021;21(1):436.
306. Mongkolpathumrat P, Nernpermpisooth N, Kijtawornrat A, Pikwong F, Chouyratchakarn W, Yodsheewan R, et al. Adeno-associated virus 9 vector-mediated cardiac-selective expression of human secretory leukocyte protease inhibitor attenuates myocardial ischemia/reperfusion injury. Front Cardiovasc Med. 2022;9:976083.
307. Nitchawat Paiyabhroma NN, Sarawut Kumphune. The Recombinant Human Secretory Leukocyte Protease Inhibitor (SLPI) protects cardiac fibroblasts injury against

an in vitro ischemia/reperfusion injury 2018. 156-62 p.
308. Mongkolpathumrat P, Kijtawornrat A, Prompunt E, Panya A, Chattipakorn N, Barrère-Lemaire S, et al. Post-Ischemic Treatment of Recombinant Human Secretory Leukocyte Protease Inhibitor (rhSLPI) Reduced Myocardial Ischemia/Reperfusion Injury. Biomedicines. 2021;9(4).
309. Mongkolpathumrat P, Kijtawornrat A, Suwan E, Unajak S, Panya A, Pusadee T, et al. Anti-Protease Activity Deficient Secretory Leukocyte Protease Inhibitor (SLPI) Exerts Cardioprotective Effect against Myocardial Ischaemia/Reperfusion. Biomedicines. 2022;10(5):988.
310. Nernpermpisooth N, Prompunt E, Kumphune S. An in vitro endothelial cell protective effect of secretory leukocyte protease inhibitor against simulated ischaemia/reperfusion injury. Experimental and therapeutic medicine. 2017;14(6):5793-800.
311. Kongpol K, Nernpermpisooth N, Prompunt E, Kumphune S. Endothelial-Cell-Derived Human Secretory Leukocyte Protease Inhibitor (SLPI) Protects Cardiomyocytes against Ischemia/Reperfusion Injury. Biomolecules. 2019;9(11).
312. Kantapich Kongpol RY, Nitirut Nernpermpisooth, Sarawut Kumphune. Recombinant human secretory leukocyte protease inhibitor ameliorated vessel preservation in experimentally isolated rat arteries2020. 107-14 p.
313. Ghasemlou N, Bouhy D, Yang J, Lopez-Vales R, Haber M, Thuraisingam T, et al. Beneficial effects of secretory leukocyte protease inhibitor after spinal cord injury. Brain : a journal of neurology. 2010;133(Pt 1):126-38.
314. Lentsch AB, Yoshidome H, Warner RL, Ward PA, Edwards MJ. Secretory leukocyte protease inhibitor in mice regulates local and remote organ inflammatory injury induced by hepatic ischemia/reperfusion. Gastroenterology. 1999;117(4):953-61.
315. Xu T, Qin G, Jiang W, Zhao Y, Xu Y, Lv X. 6-Gingerol Protects Heart by Suppressing Myocardial Ischemia/Reperfusion Induced Inflammation via the PI3K/Akt-Dependent Mechanism in Rats. Evidence-Based Complementary and Alternative Medicine. 2018;2018:6209679.
316. Pantke U, Volk T, Schmutzler M, Kox WJ, Sitte N, Grune T. Oxidized proteins as a marker of oxidative stress during coronary heart surgery. Free Radic Biol Med. 1999;27(9-10):1080-6.
317. Oran I, Oran B. Ischemia-Modified Albumin as a Marker of Acute Coronary Syndrome: The Case for Revising the Concept of “N-Terminal Modification” to “Fatty Acid Occupation” of Albumin. Disease Markers. 2017;2017:5692583.
318. Sanit J, Prompunt E, Adulyaritthikul P, Nokkaew N, Mongkolpathumrat P, Kongpol K, et al. Combination of metformin and p38 MAPK inhibitor, SB203580, reduced myocardial ischemia/reperfusion injury in non-obese type 2 diabetic Goto-Kakizaki rats. Exp Ther Med. 2019;18(3):1701-14.
319. Zabieglo K, Majewski P, Majchrzak-Gorecka M, Wlodarczyk A, Grygier B, Zegar A, et al. The inhibitory effect of secretory leukocyte protease inhibitor (SLPI) on formation of neutrophil extracellular traps. J Leukoc Biol. 2015;98(1):99-106.
320. Li H-w, Meng Y, Xie Q, Yi W-j, Lai X-l, Bian Q, et al. miR-98 protects endothelial cells against hypoxia/reoxygenation induced-apoptosis by targeting caspase-3. Biochemical and Biophysical Research Communications. 2015;467(3):595-601.
321. Kanemitsu H, Takai S, Tsuneyoshi H, Nishina T, Yoshikawa K, Miyazaki M, et al. Chymase inhibition prevents cardiac fibrosis and dysfunction after myocardial

infarction in rats. Hypertension research : official journal of the Japanese Society of Hypertension. 2006;29(1):57-64.
322. Oyamada S, Bianchi C, Takai S, Chu LM, Sellke FW. Chymase inhibition reduces infarction and matrix metalloproteinase-9 activation and attenuates inflammation and fibrosis after acute myocardial ischemia/reperfusion. The Journal of pharmacology and experimental therapeutics. 2011;339(1):143-51.
323. Zheng J, Wei CC, Hase N, Shi K, Killingsworth CR, Litovsky SH, et al. Chymase mediates injury and mitochondrial damage in cardiomyocytes during acute ischemia/reperfusion in the dog. PLoS One. 2014;9(4):e94732.
324. Hooshdaran B, Kolpakov MA, Guo X, Miller SA, Wang T, Tilley DG, et al. Dual inhibition of cathepsin G and chymase reduces myocyte death and improves cardiac remodeling after myocardial ischemia reperfusion injury. Basic Research in Cardiology. 2017;112(6):62.
325. Wahl SM, McNeely TB, Janoff EN, Shugars D, Worley P, Tucker C, et al. Secretory leukocyte protease inhibitor (SLPI) in mucosal fluids inhibits HIV-I. Oral diseases. 1997;3 Suppl 1:S64-9.
326. Lentsch AB, Jordan JA, Czermak BJ, Diehl KM, Younkin EM, Sarma V, et al. Inhibition of NF-kappaB activation and augmentation of IkappaBbeta by secretory leukocyte protease inhibitor during lung inflammation. Am J Pathol. 1999;154(1):239-47.
327. Subramaniyam D, Hollander C, Westin U, Erjefalt J, Stevens T, Janciauskiene S. Secretory leukocyte protease inhibitor inhibits neutrophil apoptosis. Respirology. 2011;16(2):300-7.
328. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. Redox Biology. 2015;6:524-51.
329. Caimi G, Canino B, Incalcaterra E, Ferrera E, Montana M, Lo Presti R. Behaviour of protein carbonyl groups in juvenile myocardial infarction. Clin Hemorheol Microcirc. 2013;53(4):297-302.
330. Dennis KE, Hill S, Rose KL, Sampson UK, Hill MF. Augmented cardiac formation of oxidatively-induced carbonylated proteins accompanies the increased functional severity of post-myocardial infarction heart failure in the setting of type 1 diabetes mellitus. Cardiovasc Pathol. 2013;22(6):473-80.
331. Bhakthavatsala Reddy C, Cyriac C, Desle HB. Role of "Ischemia Modified Albumin" (IMA) in acute coronary syndromes. Indian Heart J. 2014;66(6):656-62.
332. Maneewong K, Mekrungruangwong T, Luangaram S, Thongsri T, Kumphune S. Combinatorial Determination of Ischemia Modified Albumin and Protein Carbonyl in the Diagnosis of NonST-Elevation Myocardial Infarction. Indian J Clin Biochem. 2011;26(4):389-95.
333. Nugteren S, Samsom JN. Secretory Leukocyte Protease Inhibitor (SLPI) in mucosal tissues: protects against inflammation, but promotes cancer. Cytokine & Growth Factor Reviews. 2021.
334. Kumphune S, Chattipakorn S, Chattipakorn N. Role of p38 inhibition in cardiac ischemia/reperfusion injury. European journal of clinical pharmacology. 2012;68(5):513-24.
335. Silvestre-Roig C, Braster Q, Ortega-Gomez A, Soehnlein O. Neutrophils as regulators of cardiovascular inflammation. Nat Rev Cardiol. 2020;17(6):327-40.
336. Bolli R, Cannon RO, Speir E, Goldstein RE, Epstein SE. Role of cellular

proteinases in acute myocardial infarction. I. Proteolysis in nonischemic and ischemic rat myocardium and the effects of antipain, leupeptin, pepstatin and chymostatin administered in vivo. J Am Coll Cardiol. 1983;2(4):671-80.
337. Czarkowska PCB, Przybylski J, Marciniak A, Pawłowska M, Juskowa J, Foroncewicz B, et al. Proteolytic enzymes activities in patients after myocardial infarction correlate with serum concentration of TGF-beta. Inflammation. 2004;28(5):279-84.
338. Deten A, Hölzl A, Leicht M, Barth W, Zimmer HG. Changes in extracellular matrix and in transforming growth factor beta isoforms after coronary artery ligation in rats. J Mol Cell Cardiol. 2001;33(6):1191-207.
339. Vaughn G, Detels R. Protease inhibitors and cardiovascular disease: analysis of the Los Angeles County adult spectrum of disease cohort. AIDS Care. 2007;19(4):492-9.
340. Alvi RM, Neilan AM, Tariq N, Awadalla M, Afshar M, Banerji D, et al. Protease Inhibitors and Cardiovascular Outcomes in Patients With HIV and Heart Failure. J Am Coll Cardiol. 2018;72(5):518-30.
341. Schillaci G, Socio GVLD, Pirro M, Savarese G, Mannarino MR, Baldelli F, et al. Impact of Treatment With Protease Inhibitors on Aortic Stiffness in Adult Patients With Human Immunodeficiency Virus Infection. Arteriosclerosis, Thrombosis, and Vascular Biology. 2005;25(11):2381-5.
342. Ryom L, Lundgren JD, El-Sadr W, Reiss P, Kirk O, Law M, et al. Cardiovascular disease and use of contemporary protease inhibitors: the D:A:D international prospective multicohort study. Lancet HIV. 2018;5(6):e291-e300.
343. Doumas S, Kolokotronis A, Stefanopoulos P. Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor. Infection and immunity. 2005;73(3):1271-4.
344. Taggart CC, Cryan S-A, Weldon S, Gibbons A, Greene CM, Kelly E, et al. Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding. The Journal of experimental medicine. 2005;202(12):1659-68.
345. Chen CY, Lin YS, Chen CH, Chen YJ. Annexin A2-mediated cancer progression and therapeutic resistance in nasopharyngeal carcinoma. J Biomed Sci. 2018;25(1):30.
346. Rogulski K, Li Y, Rothermund K, Pu L, Watkins S, Yi F, et al. Onzin, a c-Myc-repressed target, promotes survival and transformation by modulating the Akt–Mdm2–p53 pathway. Oncogene. 2005;24(51):7524-41.
347. Kasperkiewicz P. Peptidyl Activity-Based Probes for Imaging Serine Proteases. Front Chem. 2021;9:639410.
348. Rawlings ND, O'Brien E, Barrett AJ. MEROPS: the protease database. Nucleic Acids Res. 2002;30(1):343-6.
349. Di Cera E. Serine proteases. IUBMB Life. 2009;61(5):510-5.
350. Maffia P, Guerrieri D, Villalonga X, Caro F, Gomez S, Tateosian N, et al. Cementoin-SLPI fusion protein binds to human monocytes and epithelial cells and shows higher biological activity than SLPI. Scientific Reports. 2018;8.
351. Hoffmann M, Quabius ES, Fabian A, Laudien M, Ambrosch P. The interaction of smoking habit, SLPI and AnxA2 in HPV associated head and neck and other cancers. Cancer Treatment and Research Communications. 2021;26:100299.
352. Woodham AW, Sanna AM, Taylor JR, Skeate JG, Da Silva DM, Dekker LV, et

al. Annexin A2 antibodies but not inhibitors of the annexin A2 heterotetramer impair productive HIV-1 infection of macrophages in vitro. Virol J. 2016;13(1):187.
353. Taggart CC, Lowe GJ, Greene CM, Mulgrew AT, O'Neill SJ, Levine RL, et al. Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. The Journal of biological chemistry. 2001;276(36):33345-52.

BIOGRAPHY

Name-Surname PODSAWEE MONGKOLPATHUMRAT

Date of Birth

Address

Education Background

2015 B.Sc.(Cardio-Thoracic Technology) with second class honour
Faculty of Allied Health Sciences, Naresuan university

2017 M.Sc. (Biomedical Sciences)
Faculty of Allied Health Sciences, Naresuan university

Publication

Poster Presentation:
March 2018 The 19th National Graduate Research Conference, Khon Khae, Thailand "The Combinatorial Effect of Metforminandp38 MAPK Inhibitor on Blood Glycemic Parameters in Non-obese Hyperglycemic Rat"

October 2018 2018 International Congress of Diabetes and Metabolism, Seoul, Korea "Internal organs inflammation in non-obese hyperglycemic rat"

Publication:
1. Mongkolpathumrat P., Nokkaew N., Adulyaritthikul P., Kongpol K., Sanit J., Pankhong P., Kumphune S. Diabetes induced internal organs inflammation in non-obese type 2 diabetic rats. J. Appl. Pharm. Sci. 2019; 9 (3): 41-49

2. Adulyaritthikul P., Sanit J., Nokkaew N., Kongpol K., Mongkolpathumrat P., Rojviriya C., na Lampang S., Kumphune S. The effect of metformin and p38 MAPK inhibitor on diabetic bone porosity in non-obese type 2 diabetic rats. J. Appl. Pharm. Sci. 2019; 9(07): 082-090

3. Sanit S., Prompunt E., Adulyaritthikul P., Nokkaew N., Mongkolpathumrat P., Kongpol K., Kijtawornrat A., Petchdee S., Barrère-Lemaire S., Nargeot J., Kumphune S. The Combination of metformin and p38 MAPK inhibitor reduced myocardial ischemia/reperfusion injury in non-obese type 2 diabetic Goto-Kakizaki (GK) rats. Exp. Ther. Med. 2019; 18 1701-1714

4. Nokkaew N., Sanit J., Mongkolpathumrat P., Boontawee S., Ithipruchyabun S., Plangklang W., Adulyaritthikul P., Kongpol K., Kumphune S. Anti-diabetic drug, metformin, and the p38 inhibitor (SB203580) reduces internal organs oxidative stress in non-obese type 2 diabetic rats. J. Appl. Pharm. Sci. 2019; 9 (2): 12-20

5. Nokkaew N., Mongkolpathumrat P., Junsiri R., Jindaluang S., Tualamun N., Manphatthanakan N., Saleesee N., Intasang M., Sanit J., Adulyaritthikul1 P., Kongpol K., Kumphune S., Nernpermpisooth N. p38 MAPK inhibitor (SB203580) and metformin reduces Aortic protein carbonyl and inflammation in non-obese type 2 diabetic rats. Indian J Clin Biochem. 2019