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Research Article

Cardioprotective Effect of Secretory Leukocyte Protease Inhibitor (SLPI) Partially Require Association with Cardiac Annexin A2 (AnxA2)

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ABSTRACT

Ischemic heart disease (IHD) is the leading cause of death globally. Novel therapeutic interventions are being developed to reduce the rates of death and disease. Previous studies have demonstrated that secretory leukocyte protease inhibitor (SLPI) has a cardioprotective effect on myocardial ischemia/reperfusion (I/R) injury. Annexin A2 (ANXA2), a negatively charged phospholipid-binding protein, has been proven to be involved in the anti-apoptotic activity of SLPI. Nevertheless, the correlation between cardiac ANXA2 and SLPI, as well as their role in the cardioprotective effects of SLPI against hypoxia/reoxygenation (H/R) injury, has not been investigated. The hypothesis of this study is that the expression of ANXA2 in ventricular myocytes and ventricular tissue, as well as the association and significance of the cardiac ANXA2-SLPI complex, play a role in protecting against *in vitro* hypoxia/reoxygenation (H/R) damage in rat cardiac myoblast (H9c2) cells. The results indicated that ANXA2 was expressed in ventricular cardiomyocytes and tissue. Immunoprecipitation analysis demonstrated that cardiac ANXA2 has the ability to interact with SLPI. Prior administration of anti-ANXA2 before treatment with recombinant human SLPI resulted in a considerable impairment of the cardioprotective efficacy of SLPI against *in vitro* H/R injury. The results were also observed in an anti-protease-deficient mutant of SLPI, indicating that the direct impact of SLPI relies on its interaction with ANXA2. This work presents novel molecular evidence supporting the link between cardiac ANXA2 and SLPI and the cardioprotective effect of SLPI against H/R damage. These findings have important implications for the potential therapeutic uses of SLPI in the future.

Keywords:
Ischemic Heart Disease; Secretory Leukocyte Protease Inhibitor (SLPI); Therapeutic Protein; Annexin A2; cardioprotection

1. INTRODUCTION

Ischemic heart disease (IHD) is a leading cause of global mortality. During myocardial ischemia and reperfusion, various biochemical processes occur, including post-ischemic inflammation, the production of oxygen radicals, polymorphonuclear cell infiltration, and protease release. These contribute to tissue damage, cell necrosis, and subsequent functional impairment[1,2]. Protease enzymes cause widespread destruction and have relatively long half-lives in tissue[3]. Moreover, an increase in the activity of proteolytic enzymes such as chymase, matrix metalloproteinases, calpains, cathepsins, and caspases contributes to the processes of cell death, injury, and cardiac remodeling, which is a major mechanism for the progression of heart failure[4]. Inhibition of protease

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activity can therefore be considered a powerful strategy for the prevention of ischemia/reperfusion (I/R)-induced tissue injury as well as the progression of cardiac hypertrophy and heart failure.

Our previous study demonstrated that giving recombinant human secretory leukocyte protease inhibitor (rhSLPI) by means of overexpression of rhSLPI gene[5-7] or treatment with recombinant protein of human SLPI provided cytoprotective effect against I/R injury both in an *in vitro*[5,8-13], *ex vivo*[8], and *in vivo*[7,14,15] study model. Interestingly, although the beneficial effects of SLPI are believed to be due to its anti-protease activity, few studies showed that those effects of SLPI might be independent of its anti-protease activity[16,17]. Recently, our study showed that recombinant proteins of anti-protease-deficient mutant SLPI (L72K, M73G, and L74G) (mt-SLPI) also provided cardioprotection against I/R injury[15]. Therefore, the therapeutic potential of SLPI is believed to be in part due to its direct effect

One of the possible explanations for the anti-apoptotic activity of SLPI is the association between SLPI and Annexin A2. Annexin A2 (ANXA2) is a negatively charged phospholipid-binding protein. It's N-terminal domain contains the binding sites for p11 protein (S100A10) and tissue-type plasminogen activator (tPA), and a C-terminal domain contains binding sites for calcium, phospholipids, and the actin cytoskeleton[18]. ANXA2 functions in various cellular pathways, including signal transduction, membrane fusion, cell adhesion, DNA synthesis, cell proliferation, and fibrinolysis[19-21] This information suggests that ANXA2 is required for cell survival. ANXA2 has been reported to express in several types of tissue and organs, including vascular endothelial cells, monocytes and macrophages, and some tumor cells[22]. AnxA2 is prominently expressed in the pancreas, lung, ileum, colon, and adrenal tissues, but it is less expressed in the liver, kidney, testis, and spleen[23]. Some studies also reported that ANXA2 could be expressed in the heart[24,25],but the actual localization of ANXA2 could not be confirmed because it actually expresses on the cardiomyocytes, and its roles and dynamics in cardiac pathology, particularly in I/R injury, have not been clarified. Moreover, the association between SLPI and ANXA2 and cardioprotection has never been investigated. The primary goals of this study were to examine the association between cardiacANXA2 and the SLPI and to determine if the interaction between cardiacANXA2 and the SLPI contributes to the cardioprotective effects of SLPI.

2. MATERIALS AND METHODS

2.1. Chemical and reagents

All basic chemicals were purchased from Sigma (Sigma, St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and trypsin-EDTA were purchased from Gibco (Gibco-Thermo Fisher Scientific, USA). CyQUANTTM LDH Cytotoxicity Assay Kit and 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2Htetrazolium bromide (MTT) were purchased from Invitrogen (Invitrogen-Thermo Fisher Scientific, USA). The wild-type recombinant human SLPI (wt-SLPI) protein was purchased from Sino Biology Inc. (Beijing, China). A protease-deficient mutant of SLPI was produced and purified according to the previous published article[15].

2.2. Cell type and cell culture

Cardiac myoblast cell line (H9c2) was purchased from American Type Cell Culture (ATCC-CRL1446) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5,000 units of penicillin, and streptomycin. Cells were cultured at 37◦C with 5% CO_2 and 95% air throughout the experiments.

2.3. Immunoprecipitation

H9c2 cells were seeded into six well plates until they reached 80% confluence. Cells were washed once with cold phosphate-buffered saline and then scraped into 1 ml of lysis buffer (20 mM Tris pH 6.8, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and cOmplete™, mini protease inhibitor cocktail tablet; Sigma-Aldrich; Merck). In addition, we also determine the presence of ANXA2 in isolated rat ventricular myocytes (ARVMs) and cardiac tissue using cells and tissue extracts from previous studies. The cells and tissue were extracted on ice and centrifuged at 15,000 RPM for 10 minutes at 4°C. Supernatants were collected for further analysis. Five microliters of rabbit polyclonal Annexin-2 antibody (Ab41803, Abcam) were diluted in 200 µl of PBS pH 7.4 with 0.02% TweenTM 20 and incubated with rotation for 10 minutes at room temperature. Then, magnetic beads were collected and washed twice with PBS. Then, 500 µl of supernatant samples (from H9c2 cells, adult rat ventricular myocytes /ARVMs, and ventricular tissue) were added and incubated with rotation for 60 minutes at room temperature to allow Ag to bind to the magnetic bead-Ab complex. Then, magnetic beads were collected and washed twice with PBS. Then, 20 µL of elution buffer and 10 µL of premixed NuPAGETM LDS sample buffer and NuPAGETM sample reducing agent were added to the reaction. The samples were boiled at 95°C for 5 minutes and separated on 12% SDS-polyacrylamide gels, followed by blotting using primary antibodies against SLPI (ab17157, Abcam) and ANXA2 (sc-28385, Santa Cruz Biotechnology).

2.4 Pre-treatment with ANXA2 antibody

H9c2 cells were seeded in 96-well plates at a density of 1 x 10^4 cells per well and incubated overnight at 37°C with 5% CO_2. Then, cells were pre-treated with 25 µg/mL of anti-Annexin-2 (Ab41803, Abcam) for 24 hours, followed by incubation with 1 µg/mL of rhSLPI or mut-SLPI for a further 1 hour. At the end of the treatment period, cells were subjected to *in vitro* hypoxia and reoxygenation (H/R).

2.5 An *in vitro* hypoxia/reoxygenation (H/R)

An *in vitro* hypoxia/reoxygenation (H/R) protocol was modified from a previous study[15]. Hypoxia was performed by overlaying the culture well with 200 µl of liquid paraffin and incubating at 37 °C for 1 hour. At the end of the hypoxic period, the overlay liquid and culture medium were replaced by a completed medium and incubated at 37 °C for 3 hours. Then, cell viability was also determined by the MTT cell viability assay.

2.6 Determination of cell viability

After treatments or at the end of study protocols, the culture medium was discarded and replaced with 0.5 mg/ml MTT reagent and incubated at 37°C for 2 hours. After incubation, the excess MTT reagent was removed, and the formazan dye was solubilized by adding 100 µl of dimethysulfoxide (DMSO). The optical density (OD) was measured using a spectrophotometric method at λ 490 nm using DMSO as a blank. The relative percentage of cell viability was compared against the control group.

2.7 Statistical analysis

The statistical tests were performed using commercially available software (GraphPad Prism). All values were expressed as Mean ± SD. All comparisons were assessed for significance using ANOVA, followed when appropriate, by the Tukey-Kramer test. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Cardiac Annexin A2 (ANXA2) interact with SLPI

To prove if AnnexinA2 expresses on cardiac cells, the protein lysate of rat cardiac myoblast H9c2, the primary culture of isolated adult rat ventricular myocytes (ARVMs), and rat ventricular heart tissue were determined for the presence of ANXA2 by Western blot analysis. The results showed that the expression of the ANXA2 protein could be detected in all types of cardiac cells and tissue lysate samples (Figure 1A).

To determine whether SLPI interacts with ANXA2, we used protein extracts from the rat cardiomyoblast cell line H9c2 and ventricular tissue extracts. The SLPI-cardiac protein complexes were precipitated, and Western blotting with an anti-ANXA2 antibody was performed to determine the presence of ANXA2 protein in the SLPI-cardiac protein complex. The results showed that both SLPI and cardiac ANXA2 could associate with ANXA2 from H9c2 lysate and heart homogenate (Figure 1B), indicating that there is a physical association between SLPI and cardiac ANXA2 protein.

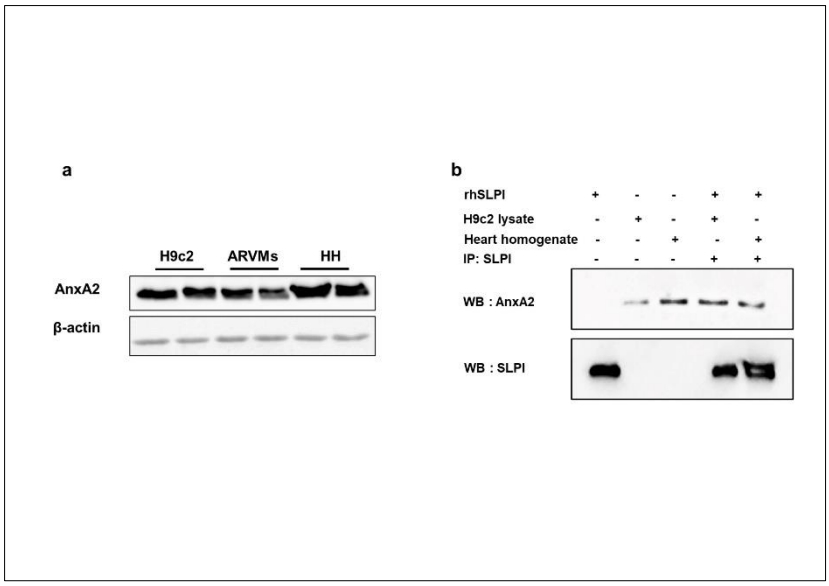

Figure 1. Western blot analysis for the expression of ANXA2 in rat cardiac myoblast cell line (H9c2), isolated adult rat ventricular myocytes (ARVMs), and rat heart homogenate (HH) (a). Co-immunoprecipitation (IP) studies of ANXA2 and rhSLPI in cardiac cell extracts (b).

3.2 Optimization of H/R induced cardiac cell death

An optimization study was conducted to determine the optimal length of hypoxia/reoxygenation (H/R) that resulted in approximately 50% cell death. This was achieved by placing a liquid paraffin cover on the surface of the culture medium to induce hypoxia for different time periods, followed by reoxygenation for 24 hours. The results demonstrated that hypoxia at 1 h resulted in a substantial decrease in cell viability, reducing it to around 50% (57.99% ± 2.47%, $p < 0.05$) compared to the control group (Figure 2A). Therefore, the H/R procedure in subsequent trials utilized a 1h of hypoxia followed by24 h of reperfusion.

3.3 Annexin A2 antibody did not cause cardiac cell injury

The effect of ANXA2 blockage on cardiac cell survival has never been reported. The results in (Figure 2B) showed that pre-treatment of 25 µg/mL of anti-Annexin-2 in the presence and absence of 1 µg/mL rhSLPI for 24 hours did not cause cardiac cell death. The percentage of cell viability was greater than 95% in each experimental group.

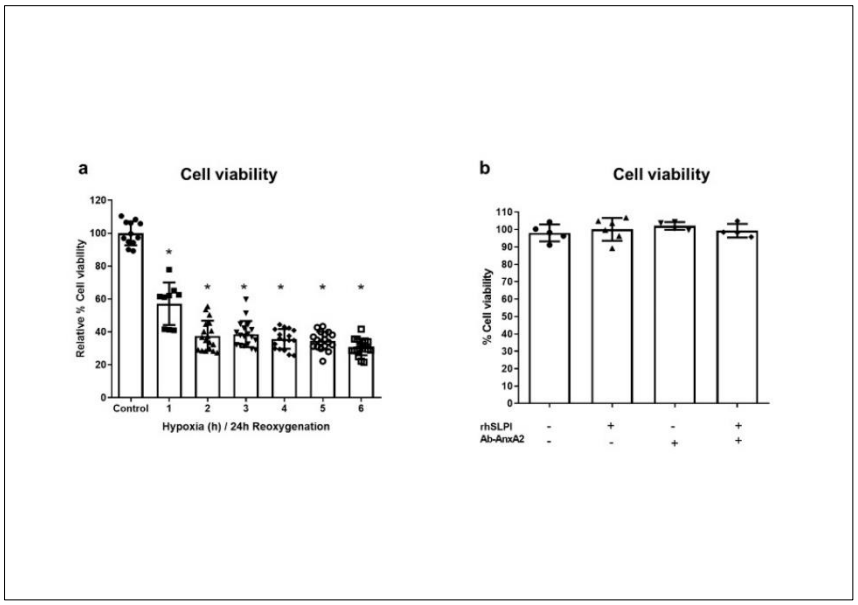

Figure 2. Optimization of hypoxia/reoxygenation protocols in H9c2 (a). The cytotoxicity of anti-ANXA2 on H9c2 cells (b).

3.4 Annexin A2 antibodies impaired cardioprotective effect of SLPI

The results in figure 3 showed that H/R injury significantly reduced cardiac cell viability when compared to control (60.50 ± 6.6% vs. 99.40 ± 5.2%, $p < 0.05$). Treatment of rhSLPI could significantly rescue cell viability from H/R injury (81.5 ± 5.9%). The specific antibody's blocking of ANXA2 significantly reduced the cytoprotective benefit of rhSLPI (70.0 ± 6.4% vs. 81.5 ± 5.9%, $p< 0.05$) (Figure 3A). Intracellular ROS was significantly increased in the H/R group when compared with the control. Treatment with rhSLPI could significantly reduce intracellular ROS levels when compared with the H/R group. Pre-treatment of anti-ANXA2 prior to rhSLPI treatment failed to reduce H/R-induced intracellular ROS production (Figure 3B).

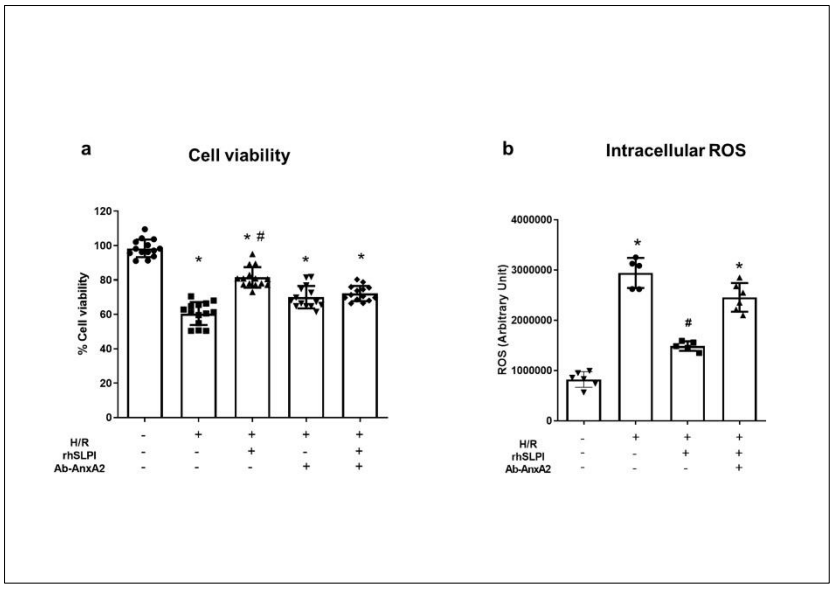

Figure 3. Determination of percentage of cell viability (a) and intracellular ROS (b) under H/R condition and treated with anti-ANXA2 in the presence and absence of rhSLPI (* $p < 0.05$ vs. control) (# $p < 0.05$ vs. H/R).

3.5 The association of Annexin A2 and SLPI is required for cardioprotection, irrespective of the anti-protease function of SLPI

We have shown, for the first time, that the anti-protease-deficient mutant SLPI (L72K, M73G, L74G, or mutant rhSLPI; mt-rhSLPI) can reduce myocardial I/R injury in cardiac cells during *in vitro* hypoxia and reoxygenation. Additionally, administration of the anti-protease-deficient mutant SLPI could also reduce the infarct size and enhance cardiac function.[15] In this study, we hypothesized that the association between ANXA2 and SLPI is required for the cardioprotective activity of SLPI, regardless of its anti-protease activity. The results showed that H/R could significantly decrease cell viability when compared with the control group (55.62 ± 7.48 % vs. 100.3 ± 8.78 %, $p < 0.05$). After treatment with m-SLPI, the result showed that mt-rhSLPI significantly increased cell viability when compared with the H/R group (74.58 ± 8.20% vs. 55.62 ± 7.48%, $p < 0.05$). Blockage of ANXA2 significantly reduced the cytoprotective benefit of mt-rhSLPI (51.31 ± 6.1% vs. 74.58 ± 8.20%, $p< 0.05$) (Figure 4A). In addition, pre-treatment of anti-ANXA2 prior to mt-rhSLPI treatment failed to reduce H/R-induced intracellular ROS production (Figure 4B).

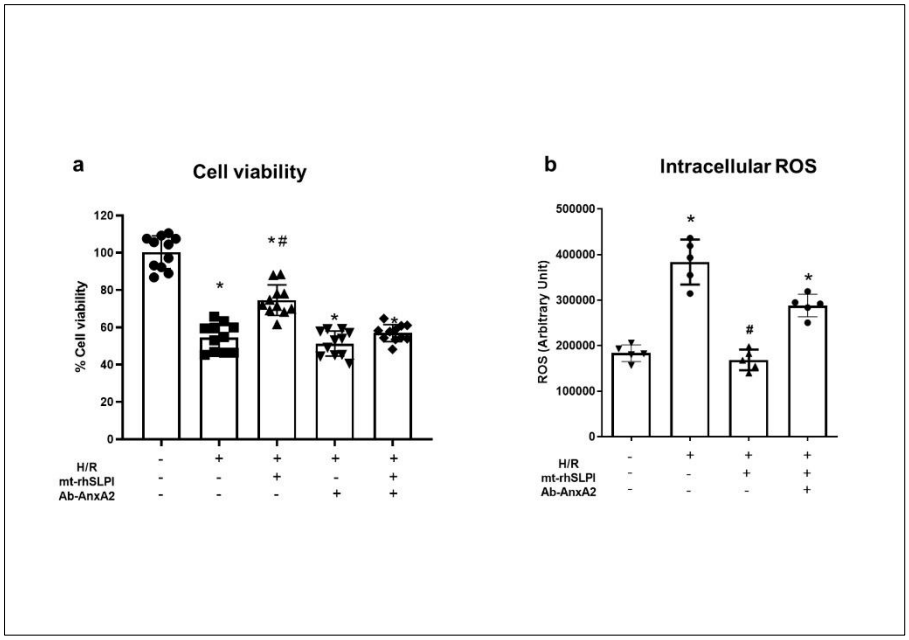

Figure 4. Determination of percentage of cell viability (a) and intracellular ROS (b) under H/R condition and treated with anti-ANXA2 in the presence and absence of anti-protease deficiency mutant rhSLPI (* $p < 0.05$ vs. control) (# $p < 0.05$ vs. H/R).

4. DISCUSSION

Secretory leukocyte protease inhibitor (SLPI) is an inflammatory serine protease inhibitor that allows for the regulation of the equilibrium between proteases and antiproteases. During inflammation, host defense effector cells create SLPI to protect tissues from harm caused by proteolytic enzymes. SLPI is not only recognized as a prominent regulator of infectious illnesses, but its involvement in non-communicable diseases (NCDs) has also been reviewed recently[26]. Among the NCDs, roles and effects of SLPI in ischemic heart disease (IHD), particularly ischemia/reperfusion (I/R) injury, have been reported, mainly from our group. Most findings highlighted the cytoprotective effect of SLPI by reducing cellular apoptosis[5-8,14,15].

Systematic residue alterations have revealed that SLPI's inhibition of serine proteases is mediated by leucine-72 (Leu72), located within a putative inhibitory loop region that extends out of domain 2[27]. Mutation at Leu72, together with the other two amino acids, Met73 and Leu74, in the inhibitory loop region, could cause SLPI to lose its ability to inhibit protease enzymes[28] Anti-protease-deficient variants of SLPI could also inhibit TNF-α- induced apoptosis by attenuating caspase-3 activation and DNA degradation[16], suggesting that the anti-apoptotic activity of SLPI is independent of its anti-protease activity. Recently, our study showed that recombinant protein of anti-protease-deficient mutant SLPI (L72K, M73G, and, L74G) (mt-SLPI) also provided cardioprotection against I/R injury. Therefore, the therapeutic potential of SLPI is believed to be in part determined by the direct effect of SLPI[15]. One of the possible explanations for the anti-apoptotic activity of SLPI is the association between SLPI and Annexin A2. Annexin A2 (ANXA2) is a negatively charged phospholipid-binding protein. It's N-terminal domain contains the binding sites for p11 protein (S100A10) and tissue-type plasminogen activator (tPA),) and a C-terminal domain contains binding sites for calcium, phospholipids, and the actin cytoskeleton[18]. ANXA2 functions in various cellular pathways, including signal transduction, membrane fusion, cell adhesion, DNA synthesis, cell proliferation, and fibrinolysis[19-21]. A previous report from Ma et al. (2004) showed for the first time that SLPI binding to the macrophage cell membrane involves ANXA2[29]. ANXA2 is found at the cell surface as the ANXA2 heterotetramer (A2t), consisting of two ANXA2 monomers and an S100A10 dimer[30,31]. Several reports showed that ANXA2 is involved in p53-mediated apoptosis[32]. Silencing of ANXA2 enhances apoptosis in various cell types[18,32-35]. Phosphorylation of ANXA2 at Tyr[23] was observed in response to various stimuli, including apoptosis. After phosphorylation at Tyr[23] through a Src-like tyrosine kinase-dependent mechanism with conformational changes, this heterotetrametric form of Annexin A2 is translocated to the cell surface[36].The ANXA2-S100A10 heterotetramer contribute to the to the subsequent stimulation of Janus kinase JAK1/TYK2 signaling. JAK1/TYK2 leads to STAT3 activation, Akt-dependent NF-kB activation, and phosphorylation of ERK1/2 and p38 MAPK[37]. This information suggests that ANXA2 is required for cell survival. Although ANXA2 is found to express in the heart[24,25], its roles and dynamics in cardiac pathology, particularly in I/R injury, have not been clarified.

It has been reported that cardiac ANXA2 is enriched in endothelial cells of intramyocardial capillaries, extracellular matrix, and coronary arteries[38]. However, determination of ANXA2 in ventricle or ventricular myocytes needs to be performed. The major findings of the current study showed that ANXA2 expresses ventricular cardiomyocytes and could form a complex with SLPI. Moreover, ANXA2 expression is reported to correlate with cell proliferation and survival, particularly in several types of cancer[39]. Knockdown of Annexin A2 has been shown to induce cellular apoptosis[40]. In this study, we showed for the first time that blockage of ANXA2 using antibodies that could interfere with the binding of SLPI significantly impaired the cardioprotective effect of SLPI against *in vitro* H/R injury and the anti-oxidative activity of SLPI. These findings were also demonstrated in an anti-protease-deficient mutant of SLPI, suggesting that the direct effect of SLPI required association with ANXA2. This study provides evidence for the first time on the interaction of SLPI and ANXA2 that is crucial for the cardioprotective activity of SLPI.

There are several issues that can be considered limitations of our study. The experimental model of blocking ANXA2 was performed in the H9c2 cells, which may not be closely related to real physiological settings in the intact heart. While H9c2 cells have biological characteristics similar to cardiomyocytes, they do not exhibit electrical features. Hence, utilizing more pertinent models, such as the augmentation of rhSLPI in the primary culture of isolated ventricular myocytes or in the intact heart, will yield more accurate functional data, as these models closely resemble the actual physiological processes occurring in the heart. Consequently, this approach may result in a more dependable interpretation. The present work primarily investigates the correlation between ANXA2 and SLPI *in vitro*, utilizing anti-ANXA2 antibody blocking. A confirmatory study might involve genetic knockdown of ANXA2 or a point mutation that disrupts the interaction between ANXA2 and SLPI. These tactics might potentially be used for an *in vivo* investigation by creating an ANXA2 knock-out or knock-in animal model in which ANXA2 is unable to bind to SLPI. Assessing the *in vivo* cardioprotective impact of SLPI

in these genetically engineered mice might yield valuable functional insights that are relevant to real-world clinical scenarios. On the other hand, this study did not provide information on signal transduction downstream of ANXA2 that explains the anti-apoptosis activity of SLPI. Further studies on the downstream signaling cascade should be considered.

5. CONCLUSIONS

This work presents the first findings on the molecular connection between cardiac ANXA2 and SLPI, as well as the cardioprotective effects of SLPI against hypoxia and reoxygenation damage. Gaining a more comprehension of the molecular mechanisms behind the activation of ANXA2 by SLPI and its subsequent signaling pathways might yield valuable data prior to advancing to clinical trials.

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Author contribution

Sarawut Kumphune conceptualized, conceived, and designed the experiments, contributed reagents/mate rials/analytical tools; Podsawee Mongkolpathumrat and Sarawut Kumphune carried out the experiments and analyzed the data, wrote, read, prepared, and approved the manuscript.

Conflict of interest

none to declare

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Ethics approval

none to declare.

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